ACTION OF FORMAMIDINE INSECTICIDES ON SPODOPTERA LITTORALIS (LEPIDOPTERA:NOCTUIDAE)

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ABSTRACT

The formamidines are a structurally novel group of pesticides with an unusual spectrum of biological activity, being effective only against ticks, mites and certain order of Lepidoptera, especially those already resistant to conventional insecticides.

Some of the behavioural, biochemical and physiological effects of formamidines and in particular chlordimeform, the prototype of this class, have been studied in the Egyptian cotton leafworm, Spodoptera littoralis. The toxicity of formamidines to different stages in the life cycle of S. littoralis is complex, with dose dependent lethal and sub-lethal effects, causing changes in reproductive and feeding behaviour which protect the plant from attack by this species.

Companion studies have attempted to find a biochemical and physiological explanation of this behaviour modification. Formamidines may interact with biogenic amines and in particular, octopamine, which in insects functions as a neurotransmitter and as a neurohormone/-neuromodulator. The distribution of octopamine has been surveyed in larval and adult moths using histochemical techniques and a sensitive radioenzymatic assay. The biochemical target does not appear to involve inhibition of amine degradative enzymes as has been suggested in some other species. Analysis of some of the principle classes of organic compounds (carbohydrates and lipids) in selected

tissues, has shown chlordimeform may mimic the neurohormonal actions of octopamine, such as stimulating carbohydrate metabolism when it is released as part of a general arousal system. This is analogous to the role of noradrenaline in vertebrates, which under stressful conditions, increases the amount of energy available for immediate use.

CONTENTS

PREFACE

Academic: Many thanks to my supervisor Dr. Denis Wright for his help and encouragement during the course of this work.

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Dedication: This thesis is dedicated to Ann Davenport.

Chapter 1

INTRODUCTION

1.1 The Formamidines

There are five types of formamidines which are of current commercial interest as insecticides and acaricides. The general structure of the groups is depicted in Fig. 1. The aromatic group is either 2-methyl, 4-chloro or 2,4 methyl substituted. R_2 can be one of five groups; examples of these are shown in Fig. 2 (Knowles, 1976).

The trisubstituted formamidines have a broad spectrum of biological activity against living organisms ranging from bacteria to insects. Chlordimeform (CDM), the prototype of the formamidines, was first synthesised in 1963 as part of a programme to develop herbicides. The novel structure and mode of action were a significant innovation which led to the development of a number of related compounds (Hollingsworth, 1976).

Introduced into the United States in 1966 under the trade name of Galecron and Fundal, the compound was subsequently withdrawn as potentially carcinogenic. It was reintroduced with improved safety precautions for use against mites and Lepidoptera (especially Heliothis species) on cotton crops and deciduous fruits. In Australia, CDM is used to control the cattle tick Boophilus microplus and the rice stem borer in Japan (Hollingsworth, 1976).

CDM acts as a stomach and contact poison. It is a base of medium strength (pK 6.8 in 50% aqueous methanol) reacting with acids to form salts. The hydrochloride (CDM.HCl) is also used in insecticidal formulations. Both compounds were used in this study.

CDM.HCl is freely soluble in water (Table 1) and the base is moderately soluble which accounts for translocation in plants and

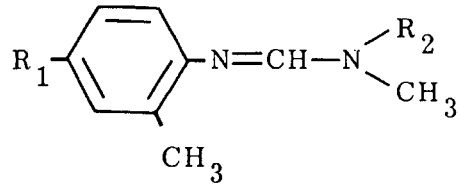
systemic toxicity to the rice stem borer. In addition, the base has a considerable vapour pressure (saturation content for air 4 mg/dm^3 at 20°C) and is an effective fumigant which may be important under field conditions. In contrast CDM.HCl has low volatility (Haddow & Shankland, 1969; Ciba Geigy, 1973; Hollingsworth, 1976).

Examples of formamidine types III and IV were also examined. Demethylchlordimeform (DCDM) is the first product of CDM degradation (Fig. 3) and is the metabolite showing the greatest biological activity (Robinson & Bittle, 1979). DCDM is not used as an insecticide or acaricide but the apparent toxicity of CDM in some organisms may be caused by conversion to this compound.

Amitraz, an example of the triazapentadienes (Type IV) has a similar commercial use to CDM against various species of Lepidoptera (particularly the eggs) and also has veterinary applications for the control of ticks and mites (Giles et al., 1979). Unlike CDM, amitraz has a low vapour pressure and solubility in water. As a result, there is no systemic activity or fumigant action.

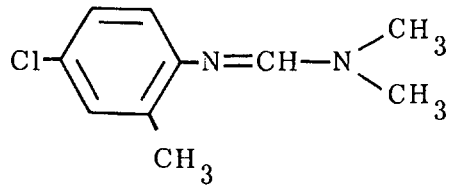
There are a number of other compounds which have a similar structure but were not examined. Hokupanon (Type II) differs from CDM only in the replacement of an N-methyl by an N-methyl-thiomethyl group. It is used exclusively against the rice stem borer and little information has been published about this compound. U-42558 (Type V) represents the sulphenylated formamidines, a recent series of compounds where the key feature is the presence of an N-sulphenyl or N-thiotrichloromethyl group in place of an N-methyl group in CDM (Parham et al., 1975). They have a similar biological activity to CDM and amitraz, but offer no insecticidal advantages. The insecticide/acaricide formetanate (NOR-AM) (Fig. 4) is both an arylformamidine and an arylcarbamate but acts as an anticholinesterase rather than a formamidine (Hollingsworth, 1976).

Fig. 1 Formamidines of current commercial interest

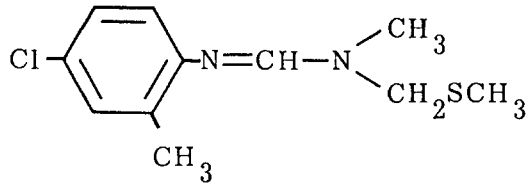


TYPE	R_2 GROUP
I	ALKYL
II	ALKYLTHIOALKYL
III	H
IV	ARYL—N=CH—
V	THIOARYL

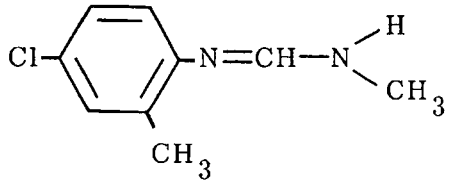
Fig. 2 Examples of the five types of formamidines



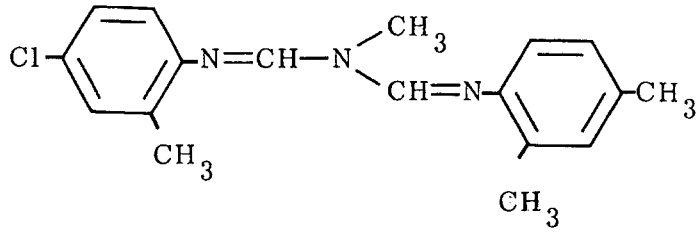
CDM



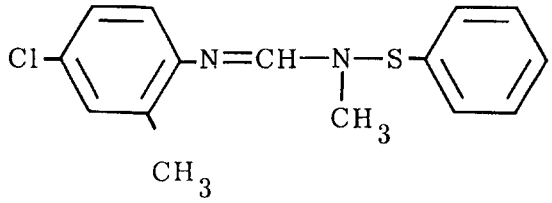
HOKUPANON



DCDM



AMITRAZ



U-42558

Fig. 3 Metabolism of CDM

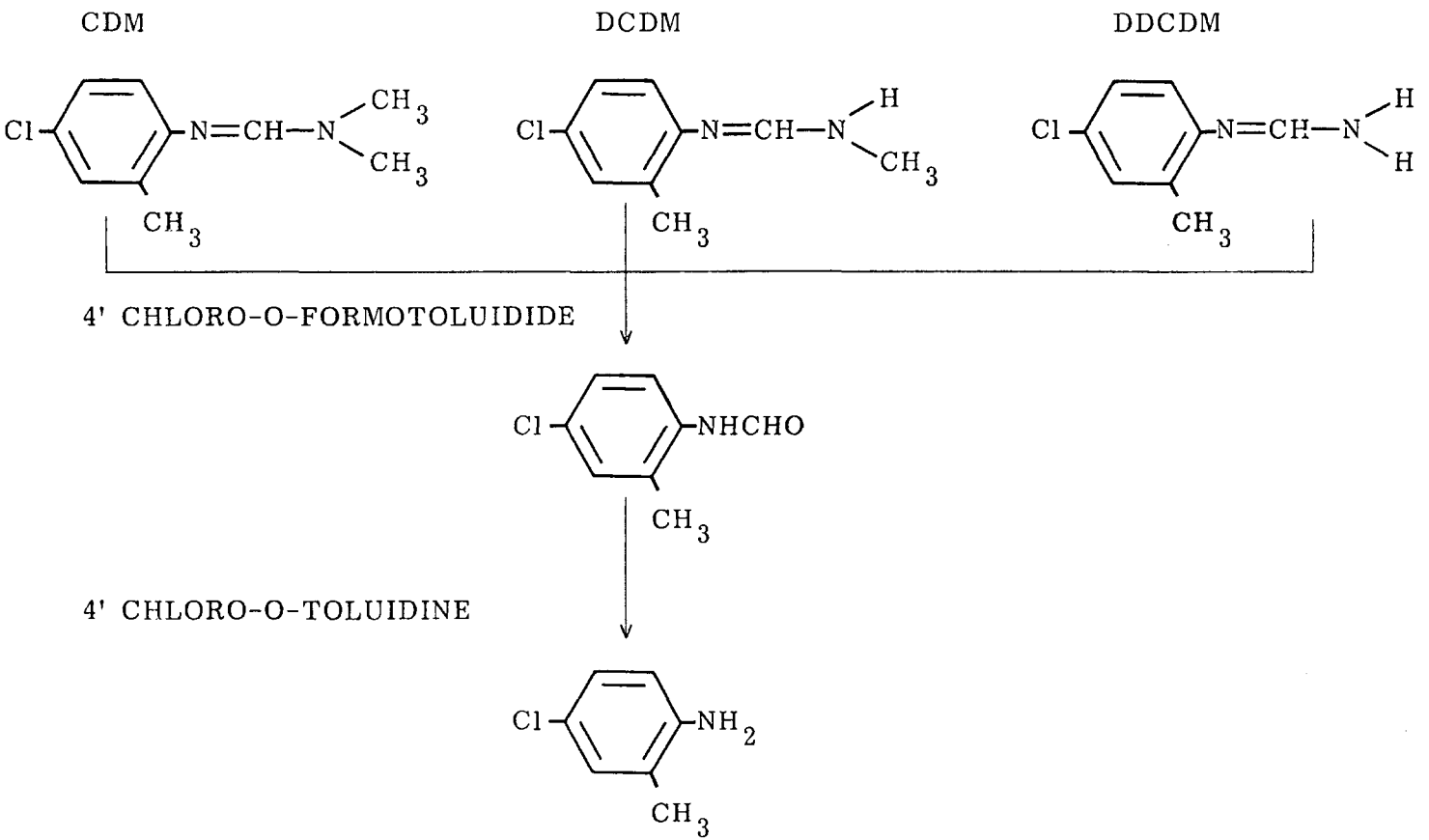
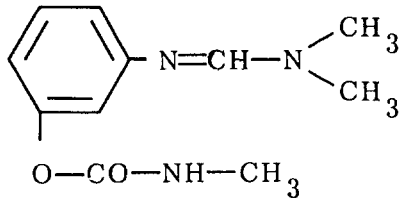
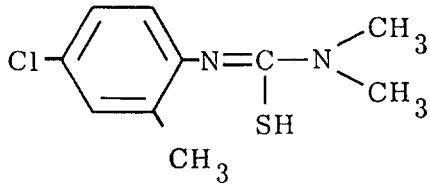


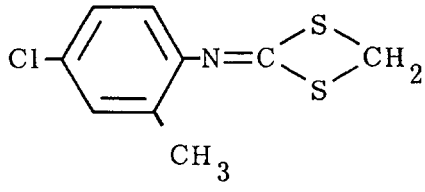
Fig. 4 Structures of pesticides related to the formamidines



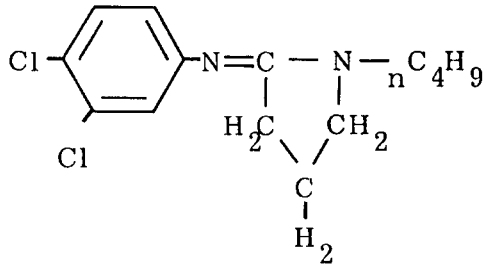
FORMETANATE



CHLORMETHIURON



ABEQUITO



CLENPYRIN

Table 1. Physical properties of the formamidines

	CDM	CDM.HCl	Amitraz
Molecular weight	196.7	233.2	293.4
Melting point °C at 1.9 x 10^3 (Pa)	32	255-227 (Decomp.)	86-87
Vapour press (Pa)	4.8×10^{-2}	2.93×10^{-5}	5.07×10^{-5}
Saturation at 20°C. mg/m^3 air	4	0.003	-
Half life in H_2O, pH 7	42 hours (30°C)	several days (20°C)	>12 weeks (20°C) unstable at acid pH
Solubility at 20°C µg/µl water	0.25	>600	0.001
Acetone >200	-	300	
Methanol	-	>300	-

Three recent pesticides (Fig. 4) which are not formamidines but have structural features and biological actions which indicate a close affinity to the group are clenpyrin (Bayer), an iminopyrrolidine; chlormethiuron (Ciba-Geigy) and abequito (American Cyamamid), an arylaminol-1,3-diethietane (Hollingsworth, 1976; Knowles, 1976).

1.2 Biological activity of formamidines

The formamidines in general, and CDM in particular, show a unique spectrum of biological activity (Tables 2,3) with remarkable selectivity to a few groups of arthropods, phytophagous mites, parasitic ticks and certain families of Lepidoptera and Hemiptera. A further general feature is their effectiveness against strains of pests which have developed resistance to conventional insecticides such as chlorinated hydrocarbons, organophosphates and carbamates. In some cases the formamidines are more active against the resistant than the susceptible strain. This occurs, for example, in mites (Dittrich, 1969) and cross resistance with CDM and lindane is negatively correlated in the rice stem borer (Hollingsworth, 1976).

CDM is often used in combination with conventional insecticides such as toxaphene, methyl parathion and azinphosmethyl (Hollingsworth, 1976). Significant synergism may occur. Plapp (1976, 1979) showed CDM enhanced the toxicity of many types of insecticide (including organophosphates, pyrethrins, carbamates and an insect growth regulator) to a resistant population of Heliothis virescens larvae. Levels of synergism ranged from 2x (methyl parathion) to 17x (pyrethrins).

The mechanism of synergism is unknown. Plapp (1976) assumed CDM acts as an alternative substrate for mixed function oxidases but Dittrich et al. (1981) found no synergism with an inhibitor of this

Table 2. Selectivity of formamidines
(From Hollingsworth, 1976)

SENSITIVE	INSENSITIVE
ACARINA:	MOST INSECTS -
PHYTOPHAGOUS &	INCLUDING PARASITES
PREDACEOUS MITES	POLLINATORS &
TICKS	PREDATORS
INSECTA:	VERTEBRATES:
LEPIDOPTERA	FISH
HEMIPTERA	BIRDS
	MAMMALS

Table 3. Selectivity of chlordimeform
(From Ciba-Geigy, 1973)

SENSITIVE	INSENSITIVE
LEPIDOPTERA	VERTEBRATES
COSMOPTERYGIDAE	FISH
GELECHIIDAE	LC_{50} 1-7 $\mu g/cm^3$ (96 H)
NOCTUIDAE	
OLEUTHREUTINAE	BIRDS
PIERIDAE	LD_{50} 1000 $\mu g/cm^3$ (8 days)
PLUTELLIDAE	
PYRALIDAE	
TORTRICIDAE	RAT ORAL LD_{50} 325 (mg/kg)
ACARINA	
ERIOPHYIDAE	
TERSONEMIDAE	
TENUIPALIDAE	
TETRANYCHIDAE	

group of enzymes and resmethrin. Synergism may result from the action of CDM at different target(s) to conventional insecticides.

With the exception of predaceous mites, populations of parasites, pollinaters and predators were not significantly reduced by application of formamidines in the field (Walters, 1976; Plapp & Bull, 1978; Sechser and Battle, 1979). For example, Ghobrial and Dittrich (1980) found conventional pesticides reduced predators of Heliothis spp. by 90% but these were unaffected by CDM. For the above reasons the formamidines appear ideal for use in integrated pest management programmes.

1.3 Direct killing action of the formamidines

The mechanism whereby formamidines protect plants and animals from arthropod attack is complex with dose-dependent lethal and sub-lethal effects, particularly at critical points in the life-cycle, such as emergence from the egg or pupae or during reproduction.

Formamidines cause various symptoms of poisoning in different organisms and also within the same organism at different stages of the life cycle. The compounds are directly toxic to the eggs, neonatal larvae and adult Lepidoptera (Dittrich, 1967; Salvisberg et al., 1980a; this study) and larvae of Acarina (Knowles & Roulston, 1973). Salvisberg and coworkers (1980a) found CDM caused increasing mortality with proceeding embryological development, suggesting certain structures must be fully formed before the insecticide has a toxic effect. Fully formed larvae still within the egg were found to be more susceptible than those which had just emerged. Removing treated embryos from their shells prior to hatching significantly reduced mortality. Although this may be a general phenomenon with other insecticides, it is particularly conspicuous with CDM. Even the apparent direct toxicity of formamidines to the egg may be due to disruption of normal eclosion behaviour which has a lethal effect.

Formamidines are moderately toxic to vertebrates, but neither CDM, amitraz or their residues are considered hazardous. Hyperactivity (the first symptom of poisoning in vertebrates) occurs within 5-10 min. with tremors to the head and limbs and episodes of convulsions. If the animal dies, it usually does so at this stage. Surviving individuals become sedated, recovering within twenty four hours (Matsumura & Beeman, 1976).

1.4. Aberrant behaviour with lethal consequences

Apart from the direct killing of neonate larvae and adults, amitraz has no effect on the later larval instars and they are relatively insensitive to CDM; yet paradoxically these pesticides are used to control arthropods in the field. How can this be achieved? A major factor is that at sub-lethal doses, formamidines can cause changes in behaviour which may have lethal consequences (Table 4).

Formamidines are known, for example, to be repellent to ticks and Lepidopterans (Beeman & Matsumura, 1978a). Death from starvation or dessication may result from the primary repellent action. In other cases, apparent toxicity is produced by a reduction in feeding - both by an anti-feeding action, where an organism refuses to eat food containing the insecticides but readily eats untreated food, or by "anorexia", where food consumption is reduced by appetite loss following treatment of the insects, but not the food. Beeman and Matsumura (1978b) have reported anorexic effects in cockroaches and CDM is an antifeedant to Hemipterans (Hirata & Sogawa, 1976). In contrast, low doses of this compound induce voracious feeding in unstarved rats representing a new class of appetite stimulants (Pfister et al., 1978b).

Hyperactivity and/or sedation is another characteristic symptom of formamidine poisoning in cockroaches, mites, ticks and Lepidopterans.

Table 4. Effects of formamidines on insect behaviour.

Species	Effective Concentration	Reference
(I) Effects on feeding: antifeedant action.		
Lepidoptera		
Bombyx mori larvae	Reduction in feeding caused by dipping leaves with 10µg/cm³ CDM.HCl	Nakamuta & Saito (1977)
Heliothis armigera larvae	Reduction in feeding on leaf discs by CDM.HCl LC_{50} = 0.14% (wt/vol.)CDM	Clift (1979)
Heliothis zea larvae	Reduction in feeding following a cycle of initial feeding hyperactivity and detachment from food source LC_{50} = 0.005% µg/cm³CDM	Lund *et al.* (1979a)
Ennomos subsignarius larvae	Reduction in feeding following field trial spraying with CDM.HCl	Doane & Dunbar (1973)
Hemiptera		
Five species	40-60% reduction in feeding by application of 10 µg/cm³ CDM to leaves	Hirata & Sogawa (1976)
(II) Effects of feeding: anorexia		
Lepidoptera		
Manduca sexta 2nd Instar larvae	Reduction in frass following injection by 0.05-1.0 µg/g CDM	Lund *et al.* (1979a)
Blattaria		
Periplaneta americana	Reduction in food intake following injection of 1-5 µg CDM	Beeman & Matsumura (1978b)
(III) Effects on feeding: appetite stimulant		
	Voracious feeding induced by injections of 40 µg/g CDM in non-food deprived rats	Pfister & Yim (1977, 1978) Pfister *et al.* (1978b) Yim *et al.* (1978)

Species	Effective Concentration	Reference
(IV) Repellency/Hyperactivity		
Acarina *Boophilus microplus*	Colony dispersal of gregarious species by 0.05% (wt/vol.) CDM	Atkinson & Knowles (1974)
Lepidoptera *Leucania separata* 3rd Instar larvae	Repellency revealed by choice bioassay using 1 µg/g CDM	Watanabe & Fukami (1977)
Ennomos subsignarius	Repellency observed in field trial with CDM.HCl (concentration unknown)	Doane & Dunbar (1973)
Hemiptera *Chilo suppressalis*	Repellency observed above 5 $\mu g/cm^3$ CDM	Hirano *et al.* (1972)
Acarina *Tetranycus* spp.	Increased "walk off" and "spin down" of adult mites from CDM treated leaves	Gemrich *et al.* (1976)
Boophilus microplus Adults	Detachment of feeding parasitic ticks from host. EC_{50} = 0.65 µg CDM/tick	Stone *et al.* (1974) Atkinson & Knowles (1974)
Nymphs and adults of 3 tick species	Detachment of feeding ticks by topical application of 0.3 - 1.0% (wt/vol.) CDM	Gladney *et al.* (1974) Dawkins & Gladney (1978)
(V) Reduction in fecundity and fertility		
Acarina *Boophilus microplus*	Decreased egg production and viability by topical application 0.01-0.2% (wt/vol.) CDM	Knowles & Roulston (1973)
Lepidoptera *Heliothis virescens*	Reduced fertility and fecundity in adults after incorporating 0.25% (wt/vol.) CDM.HCl in diet	Wolfenbarger *et al.* (1974)

This action would be very important if the pest was dislodged from the food source and unable to relocate the host. After initial feeding on CDM treated leaves, Leucania separata (Watanabe & Fukami, 1977) and Manduca sexta (Lund et al., 1979a) larvae became hyperactive and were dislodged from the food source. After spending some time relocating the leaf and starting to feed, the cycle is repeated. The net result is failure to feed adequately. Similar hyperactivity has been reported in the field. After spraying with CDM, the majority of the elm span worm Ennomos subsignarius population had fallen from the crop to the ground underneath (Doane & Dunbar, 1973).

The protection of plants and animals by formamidines thus arises partly from their direct killing but more importantly by inducing abnormal behaviour at sub-lethal doses leading to erratic mating, reduced feeding and dispersal from the host. The implications for chemical control of insects are clear: insecticides need not be lethal to be effective and behaviour modifying "pestistatic" chemicals are viable alternatives to directly lethal compounds.

1.5 Biochemical actions of CDM

A wide range of biochemical and neurophysiological actions have been attributed to CDM (Tables 5 & 6). However, despite intense examination by a number of research groups, at the beginning of the present investigation there was no general agreement regarding the nature of the biochemical target. Furthermore, as Table 5 indicates many effects were observed at high concentrations of pesticide (for example respiratory uncoupling, 10^{-3}M CDM) and in preparations from non-target species (for example, mammalian tissue culture). Often no distinction was made between physiological and pharmacological effects with little attempt to relate these actions to the symptoms of poisoning. Thus, when Abo-Khatwa and Hollingsworth (1972, 1973) reported CDM

Table 5. Biochemical and pharmacological actions of chlordimeform.

Action	Preparation	Effective Concentration	Reference
N-acetyltransferase Inhibition	Periplaneta americana nervous tissue	$I_{50}=6.0\times10^{-5}$M	Nishimura et al. (1975)
		$I_{50}=4.4\times10^{-4}$M	Matsumura & Beeman (1976)
	Locusta migratoria brain tissue	Topical application 1.2 mg/locust	Allais et al. (1979)
MAO inhibition	Rat liver	$I_{50}=1.4\times10^{-5}$M	Aziz & Knowles (1973)
	Rat liver	$I_{50}=3.2\times10^{-5}$M	Beeman & Matsumura (1973)
	Mouse liver	$I_{50}=4.7\times10^{-5}$M	Hollingsworth et al. (1979)
	Rat brain	$I_{50}=6.0\times10^{-5}$M	Benezet & Knowles (1976)
	Cattle ticks	$I_{50}=2.3\times10^{-5}$M	Atkinson et al. (1974)
Prostaglandin synthetase inhibition	Bovine	$I_{50}=3.4\times10^{-5}$M	Yim & Hollingsworth (1978)
	Seminal vesicle Microsomes	[Amitraz $I_{50}=8.8\times10^{-4}$M]	Holsapple & Yim (1979)
DNA, RNA, protein synthesis inhibition	Human cell culture	$I_{50}=1\times10^{-3}$M	Murakami & Fukami (1974)
Respiratory uncoupling	Rat liver mitochondria	$I_{50}=9.0\times10^{-5}$M	Abo-Khatwa & Hollingsworth (1973)
	Blattella germanica mitochondria	$I_{50}=6.0\times10^{-5}$M	Abo-Khatwa & Hollingsworth (1972)
	Spodoptera littoralis egg mitochondria	1×10^{-3}M	Kotter (1978)

Local anaesthetic-like actions			
Blocked compound action potentials, increased threshhold and prolonged refactory period	Manduca sexta Motor nerve	Threshold=10^{-3}M	Lund et al. (1979c)
	Frog sciatic nerve	$ED_{50}^{15}=1.5x10^{-2}$M	Chinn et al. (1976) Pfister et al. (1978a)
Similar actions to local anaesthetic lidocaine	Rat lateral ventricles (CNS)	300-1000 µg by injection	Chinn et al. (1977)
Decreased cardiac and smooth muscle contractility	Dog artery	1-30 µg by injection	Lund et al. (1978a)
	Rabbit artery	200 µg by injection	Beeman & Matsumura (1974)
Effect on calcium homeostasis			
Reduction in plasma calcium by 15% 30 min after injection	Rat	100 µg/g by injection	Pento et al. (1979)
Rapid relaxation of agonist induced contraction by reducing availability of loosely bound extracellular calcium	Rabbit aortic muscle	2 x 10^{-3} g/cm^3	Zelenski et al. (1977)
Effect on glucose homeostasis			
Increase in plasma glucose after injection	Rat	100 µg/g by injection	Pento et al. (1979)
	Mouse	3-10 µg/g by injection	Yau et al. (1977)
Effect on glucose homeostasis			
Blockage of glucose stimulated insulin release from pancreas	Isolated mouse	5 x 10^{-7}M	Yau et al. (1977)

Table 6. Neurophysiological actions of formamidines.

Action	Preparation	Effective concentration	Reference
Mimicry of the action of octopamine interacting with characterised octopamine receptors	Locust extensor tibae muscle *Schistocerca americana gregaria*	Half maximal % increase in neurally evoked contraction and relaxation rate DCDM=$5x10^{-6}$M CDM=$5x10^{-5}$M	Evans & Gee (1980)
Partial agonist of specific octopamine stimulated adenylate cyclase	Particulate preparation from light organ of adult *Photinus pyralis*	DCDM K_a=$2.2x10^{-6}$M	Nathanson & Hunnicutt (1981)
Stimulation of lantern to glow	Adult firefly lantern *Photinus pyralis*	Topical application to firefly light organ CDM 20-µg/g DCDM 100pg/g	Hollingsworth & Murdock (1980)
Induced release of hyperlipemic hormone (AKH) by action on post-synaptic aminergic receptors	Isolated locust corpus cardiacum *Locusta migratoria*	Effective concentration $5x10^{-6}$ - $5x10^{-7}$M	Singh *et al.* (1981)
Stimulation of motor nerves by action at non-cholinergic synapses	Isolated abdominal ganglion *Manduca sexta*	Threshold = 1×10^{-7}M	Lund *et al.* (1979b)
Increased repetitive discharge in the nerve cord	Ventral nerve cord *Periplaneta americana*	Threshold = 1×10^{-3}M	Beeman & Matsumura (1974)

Action	Preparation	Effective concentration	Reference
Decreased muscle end-plate sensitivity to acetylcholine	Frog sartorius muscle	Threshold = 1×10^{-4}M	Wang et al. (1975)
Decrease in acetylcholine induced contraction	Frog rectus abdominus muscle	Threshold = 1×10^{-5}M	Watanabe et al. (1975)
Depression of excitatory post synaptic potentials to glutamate and nerve stimulation	Foregut nerve muscle system of Galleria mellonella	Threshold = 2×10^{-3}M	Yamamoto & Fukami (1976, 1979)
Induction of muscle contraction	Frog rectus abdominus muscle	Threshold = 1×10^{-3}M	Watanabe et al. (1976)
Increase in spike frequency	Tarsal motor nerve Locusta migratoria	Effective concentration = 5×10^{-5}M	Salvisberg et al. (1980b)

uncoupled oxidative phosphorylation in rat liver and cockroach mitochondria with a potency similar to 2,4,dinitrophenol, and was assumed to be of some importance. While Kotter (1978) confirmed respiratory uncoupling by CDM and DCDM at high concentrations in the early stages of development of Spodoptera littoralis eggs, formamidines were only toxic to eggs just before hatching. In addition, the symptoms of hyperactivity observed in adults and larvae could not be explained by respiratory uncoupling alone.

The reported inhibition of prostaglandin synthetase may be important and could contribute to effects of formamidines on fecundity. Inhibition of this enzyme has been shown to reduce fecundity in crickets; oviposition being stimulated by post copulatory biosynthesis of prostaglandins (Yim et al., 1978).

Some common physico-chemical property of CDM may be disturbing a membrane dependent process, which could account for uncoupling of electron transport, and for inhibition of DNA, RNA, and protein synthesis.

At high concentrations, CDM may have a number of non-specific effects on membrane stability. This category includes similar actions to local anaesthetics such as the decrease in cardiac and smooth muscle contractility in mammals (Lund et al., 1978a); decrease in end plate sensitivity to acetylcholine in frogs (Wang et al., 1975) or glutamate in Lepidoptera (Yamamoto & Fukami, 1976) and the decrease in axonal transmission in the frog sciatic nerve (Chinn et al., 1976) and Lepidopteran nerves (Lund et al., 1979c).

In vertebrates, lethal doses of CDM cause cardiovascular and respiratory collapse (Beeman & Matsumura, 1974). The symptoms are similar to poisoning by local anaesthetics such as lidocaine and phenocaine, which have a structural similarity to CDM (Pfister et al., 1978a). Lund et al. (1978a, 1978b) found depression of blood

pressure by CDM was caused by a non-specific direct action on cardiac and vascular smooth muscle. This action was independent of the autonomic nervous system. Local anaesthetics have a general depressant activity on nerve and muscle which was mimicked by CDM in a number of isolated preparations (Chinn et al., 1976, 1977; Lund et al., 1978b; Pfister et al., 1978a). CDM toxicity was increased by local anaesthetics and antagonised by anticonvulsants.

Local anaesthetics compete with calcium for binding sites on excitable membranes. Calcium ions stabilise the membrane and must be displaced by a depolarizing current before the sodium channels open. If this is prevented, conduction is blocked. In skeletal muscle, local anaesthetics compete with calcium at the sarcoplasmic reticulum, increasing outflow of calcium ions and preventing reuptake (Bowman & Rand, 1980).

A similar mechanism could explain the blockage of compound action potentials by CDM in vertebrate and invertebrate motorneurones (Chinn et al., 1976). Watanabe et al. (1975, 1976) reported 10^{-5}M CDM decreased acetylcholine induced contractions of the frog rectus abdominus muscle, which was correlated with the toxicity of formamidines to frogs. Zelenski and co-workers (1978) showed CDM antagonised the drug-induced contraction of smooth muscle by reducing the availability of loosely bound extracellular calcium to the muscle membrane, providing further evidence of the similarity between CDM and local anaesthetics.

There is good evidence that acute poisoning in vertebrates by CDM is caused by cardiovascular collapse through actions on muscle and nerves which are common to local anaesthetics. Similar local anaesthetic-like actions have been observed in invertebrates but only at high concentrations and there is no evidence yet that they contribute to the toxicity of formamidines.

In contrast to the sub-lethal reduction of feeding in insects, formamidines stimulate appetite when injected into rats (Pfister et al., 1978b; Yim et al., 1978). In mammals the sensation of hunger is regulated by structures containing biogenic amines (Soulairac, 1969). Serum glucose and insulin also modulate appetite. Yau et al. (1977) and Pento et al. (1979) demonstrated sub-lethal doses of CDM produce hyperglycaemia (a 64% rise in plasma glucose was observed) in fasted and also in fed animals. Increased food consumption did not therefore cause the hyperglycaemia.

Changes in carbohydrate homeostasis were caused by decreased pancreatic responsiveness to glucose, reducing insulin release. Exposure of isolated pancreatic islets to 5×10^{-7}M CDM reduced glucose stimulated insulin release by 50%. Although Pento and co-workers (1979) suggested the parallel elevation of plasma glucose produced by Monoamine oxidase inhibitors may, in both cases, be the result of an increase in circulating catecholamines, they did not rule out the possiblity of some other mechanism.

Watanabe et al. (1975) reported 10^{-5}M CDM decreased acetylcholine induced contractions of the frog rectus abdominus muscle. Higher concentrations (10^{-3}M), caused contractions by depolarizing the muscle membrane but this was a non-specific effect unrelated to toxicity. With this exception and the report by Wang et al. (1975) which has already been mentioned, no other actions of the formamidines on the cholinergic system have been described for vertebrates or invertebrates. No evidence has been found for anticholinesterase activity (Dittrich, 1967; Hirano et al., 1972) or for cholinergic stimulation (Beeman & Matsumura, 1974).

Recent evidence suggests the most potent action of the formamidines is in disrupting the action of biogenic amines as invertebrate chemical messengers.

Early studies reported the formamidines were unique amongst pesticide groups in inhibiting Monoamine oxidase (MAO) from rat liver (Aziz & Knowles, 1973), rat brain (Laureut et al., 1978; Maitre et al., 1978), mouse liver (Beeman & Matsumura, 1973) and cattle tick (Atkinson et al., 1974). Inhibition of this enzyme is unlikely to be significant in most insects because with the exception of Malpighian tubules, MAO is not readily detected in insect tissue. Inhibition of other inactivating enzymes such as N-acetyltransferase, may be more important as this enzyme is widely distributed in insects (Evans & Fox, 1975).

Subsequent reports showed inhibition of MAO was not an important target in vertebrates and acarines. The symptoms of poisoning by MAO inhibitors were completely different to the rapid onset of excitation and tremors of CDM poisoning (Matsumura & Beeman, 1976). Neuman and Voss (1977) found in vivo MAO inhibition was not a structure specific phenomenon and there was no correlation between MAO inhibition potency and acute toxicity in mice. Injection of a lethal dose of CDM caused only a moderate and reversible inhibition of MAO, whereas established inhibitors of this enzyme considerably exceeded this level of inhibition, without apparent symptoms of toxicity (Hollingsworth et al., 1979). Depletion of brain noradrenaline levels had no effect on the toxicity of CDM to rats (Robinson et.al, 1975; Robinson & Smith, 1977). MAO inhibition could not be related to any symptoms of poisoning in arthropods such as tick detachment (Stone et al., 1974) or excitation in Lepidopterous larvae (Watanabe & Fukami, 1977).

Since the present study commenced knowledge of the interaction between formamidines and biogenic amines has increased substantially, suggesting formamidines are octopamine receptor agonists. This is significant because no other insecticide has been reported to interfere with octopamine or indeed other biogenic amine systems.

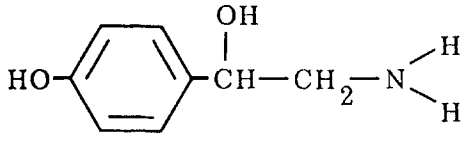
Octopamine is a phenolamine that is widely distributed within the insect nervous system (Robertson & Steel, 1974; Robertson, 1976; Evans, 1979b; Dymond & Evans, 1979; Evans, 1980a) at levels exceeding those of catecholamines. The amine is also present in non-nervous tissue (David & Lafon-Cazal, 1979; Goosey & Candy, 1980; Orchard et al., 1981). There is a structural similarity between the formamidines and phenolamines (Fig. 5).

The physiological role of octopamine is still uncertain but evidence is accumulating that the compound may function as a neurotransmitter, neuromodulator and neurohormone. A neurotransmitter can be defined as a chemical messenger released at special zones of intercellular contact, the synapse, which diffuses across the narrow cleft to act on the post synaptic membrane. At the other extreme of the spectrum for the transfer of information by chemical messengers are neurohormones, which are released into the circulatory system to be carried some distance to one or more target sites. A neuromodulator is a special type of neurohormone that changes the quality of information being passed through a conventional synapse or changes the spontaneous activity of a receptive neurone or muscle (Evans, 1980a).

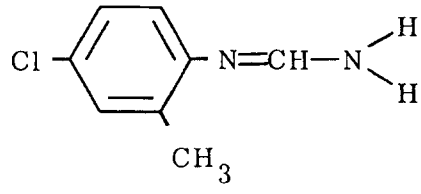
Octopamine may act as a neurotransmitter in the firefly light organ and at the synapse between nervous corpus cardiacum II (NCC II) and cells releasing hyperlipaemic hormone in the locust corpora cardiaca (Orchard & Loughton, 1980). Octopamine is a neuromodulator at insect skeletal neuromuscular junctions (Evans & O'Shea, 1977, 1978) and a potential neurohormone, stimulating carbohydrate and lipid metabolism (Orchard, 1982). The compound may be part of a general arousal system analogous to the function of noradrenaline and adrenaline in vertebrates. Release of this amine during stress would increase the magnitude of the response in skeletal muscle and the amount of energy available for immediate use (Evans, 1980a).

Fig. 5 Structures of formamidines and phenolamines

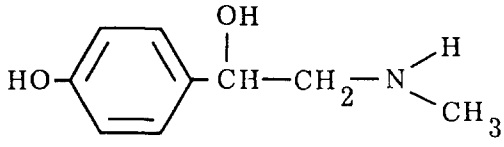
OCTOPAMINE



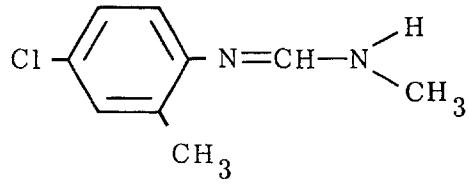
DDCDM



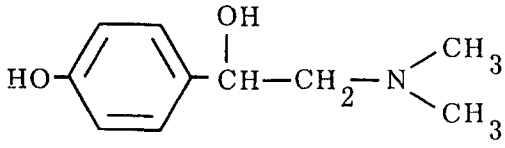
SYNEPHRINE



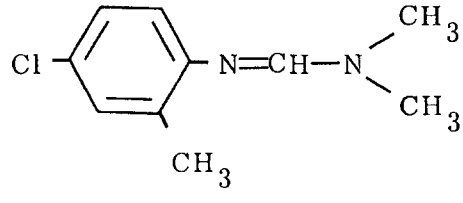
DCDM



N,N-DIMETHYLOCTOPAMINE



CDM



Early studies reported octopamine and chlordimeform (as well as many other compounds) increase the rate of beating of cockroach hearts (Beeman & Matsumura, 1974) and both compounds reduced food consumption when injected into cockroaches (Beeman & Matsumura, 1978b).

In a recent series of papers, Lund and co-workers (1979a, 1979b, 1979c) found physiological concentrations of formamidines stimulated certain unidentified motor nerves in the isolated abdominal ganglia of fourth instar Manduca sexta larvae, by their action on the post-synaptic membrane of non-cholinergic synapses. Octopamine was one of several compounds which mimicked this action. Furthermore, activation of these neurones could be correlated with increased locomotor activity and uncoordination produced by sub-lethal doses. CDM may also stimulate octopamine receptors in the adult. The activity of certain flight muscles may be modulated by octopamine neurones with cell bodies in the thoracic ganglia. Both octopamine and CDM augment excitatory function potentials recorded from pharate adults of M. sexta (L.W. Klaasen, personal communication, cited by Beeman, 1982).

The role of octopamine in the insect central nervous system has not been extensively studied and the mode of action of CDM in stimulating neurones in the central nervous system is uncertain.

When CDM was applied topically (1 µg/insect, approx. 20 µg/g) to adult male fireflies, Photinus pyralis, the light producing organs emitted a weak glow within a few minutes. After a time lag of 3-5 hours, this increased in intensity, then declined. In contrast, octopamine, DCDM and DDCDM caused continuous intense light production immediately. DCDM was most active. As little as 5 ng/insect produced a response. Two non-formamidine metabolites of CDM had no effect (Hollingsworth & Murdock, 1980). These authors suggested the delay by the parent compound in stimulating light

emission may be related to the conversion of CDM in vivo to the more potent DCDM. A number of other reports (see for example, Holden & Hatfield, 1975; Knowles & Roulston, 1973) have shown metabolic transformation of CDM is necessary to produce toxicity in ticks, although there is no evidence yet for octopaminergic actions of formamidines in acarines.

In P. pyralis, the sixth and seventh abdominal segments contain light emitting organs which are innervated by non-cholinergic neurones. Octopamine is thought to be the neurotransmitter, stimulating a specific adenylate cyclase system, but evidence for this is incomplete. Synephrine, followed by octopamine were the most potent of a series of biogenic amines inducing light production in larval fireflies (Carlson, 1968). This action was antagonised by α-adrenergic blocking agents (yohimbine and phenoxybenzamine) which were more effective than β -blockers (Smalley, 1965). Segments containing light organs are rich in octopamine but not synephrine (Robertson & Carlson, 1976) and an octopamine sensitive adenylate cyclase system has been demonstrated (Nathanson, 1979). Therefore, in this preparation octopamine has a similar action to neurotransmitters which mediate their actions on the post synaptic cells through stimulation of the enzyme adenylate cyclase.

Pharmacological studies demonstrated DCDM (CDM was not tested) had a similar effect to octopamine, and was acting post synaptically to induce light emission (Hollingsworth & Murdock, 1980). This action of DCDM could be blocked by cyproheptadine under conditions which also antagonised octopamine. They also found that DCDM stimulated adenylate cyclase activity with a potency similar to octopamine.

Nathanson and Hunnicutt (1981) extended these studies using a very active octopamine sensitive adenylate cyclase present in broken cell preparations from adult firefly light organs. At low concentrations DCDM was a partial agonist (70% of octopamine V_{max}) binding

specifically and reversibly to receptors and stimulating adenylate cyclase activity with a potency 6 times that of octopamine. α-adrenergic antagonists blocked this activity. The authors concluded DCDM and octopamine were competing either for the same site or two sites with similar characteristics. In contrast, CDM was a weak octopamine agonist (K_a = 3 x 10^{-5}M; 9% of octopamine V_{max}) which agrees with Matsumura and Beeman (1976) who found CDM had no effect on an octopamine sensitive adenylate cyclase in the cockroach thoracic ganglia. At higher concentrations, Nathanson and Hannicutt (1981) found CDM antagonised octopamine, almost completely inhibiting octopamine stimulated adenylate cyclase activity but this was a non-specific effect. Neither CDM or DCDM stimulated adrenaline or dopamine sensitive adenylate cyclases found in other tissue.

Incubation of isolated corpus cardiaca from the locust Locusta migratoria in saline containing CDM and DCDM produced hyperlipemia when the medium was injected into adult locusts (Singh et al., 1981). These authors stated the formamidines induced the release of hyperlipemic hormone which could be blocked by α-adrenergic but not β-adrenergic receptor antagonists. CDM and DCDM also potentiated the release of hyperlipemic hormone from the corpus cardiacum following electrical stimulation of NCC II. They concluded that the formamidines or their metabolites were interacting with post synaptic octopamine receptors at the synapse between NCC II and the cells of the glandular lobe releasing hyperlipemic hormone. Although Orchard and Loughton (1981) demonstrated that the neurotransmitters at this synapse is an amine, evidence that it is octopamine is still incomplete.

In the metathoracic ganglion of the locust, Schistocerca americana gregaria, an identified octopamine neurone (DUMETi) has been described, an acronym for Dorsal Unpaired Median Neurone, projecting only to the Extensor Tibiae muscle (Evans, 1980a). The pharmacology

of the octopamine receptors at this neuromuscular junction has been characterised (Evans, 1980b).

Using this preparation, Evans and Gee (1980) provide the clearest evidence that formamidines interact with octopamine receptors, having quantitively similar actions to octopamine but with a much longer time course.

Pulses of CDM superfused over the extensor tibiae muscle potentiate the rate of neurally evoked contractions by 10% and increased the rate of relaxation by 90%. Equivalent pulses of octopamine increase these two parameters by 23% and 160% respectively. Dose response curves were parallel, with no significant differences between the CDM and octopamine but DCDM was one order of magnitude more effective than CDM. This was in agreement with the rank order of potency of phenolamines where synephrine (the N-methyl analogue of octopamine) was more effective than N,N-dimethyloctopamine. The results are similar to those from the firefly light organ with phentolamine blocking the action of DCDM, CDM and octopamine. The formamidines produced longer lasting effects, possibly because of their greater tissue solubility, together with an increased half time of receptor occupancy (Evans & Gee, 1980).

The aim of the investigation was to study some of the physiological, biochemical and behavioural effects of the formamidines in the Egyptian Cotton Leafworm Spodoptera littoralis and relate some of these effects to the biogenic amine system of this insect. However it was not expected the study would reveal the ultimate mode of action of this novel insecticide group. Although little information has been published about the physiology of S. littoralis, this species was chosen as it is an actual target organism for the formamidines, unlike many used in previous studies and belongs to the family Noctuidae which includes some of the most destructive pests to world agriculture.

Chapter 2

GENERAL MATERIALS AND METHODS

2.1.1 Insect culture: Spodoptera littoralis (Boisduval)

Fourth instar larvae were periodically obtained from I.C.I. Plant Protection Ltd., Jealotts Hill, where 2000-3000 test insects were produced daily. The culture used principally was the insecticide resistant "Cairo" strain originating from the Unit of Invertebrate Virology, Oxford. A susceptible strain was also obtained from the same source.

The insects were reared under optimum conditions at 25 ± 1 °C, 70-80% R.H. and a LD 12:12 cycle. All behavioural and toxicological experiments were carried out under these conditions. A serious disadvantage in using S. littoralis and many other noctuids is their susceptibility to nuclear polyhedrosis virus. Sterilization of all containers was necessary to reduce mortality.

The culture procedure was as follows:

Eggs were sterilised with 10% (v/v) formaldehyde solution for 20 min. then washed in distilled water, dried and placed on a strip of artificial diet (Appendix 1) to hatch. Late first instar larvae were transferred into plastic boxes (28 x 16 x 19 cm) lined with polythene and cellulose wadding to absorb moisture. At the fourth instar stage, larvae were reduced to thirty per box. Pupae were removed from the cellulose wadding, sterilised in 10% (v/v) formaldehyde solution for 20 min. and sexed. Fifty male and female pupae were placed in cages covered with nylon netting and lined with paper towelling to provide oviposition sites. After emergence adults were fed on a 10% (wt./vol.) honey solution.

2.1.2 Life cycle at 25°C

Adults mate on the first night of emergence. Both sexes can mate several times, but one spermatophore is sufficient for a female to lay fertile eggs throughout the life span. Up to 1000 eggs are laid in large clusters on the underside of the leaf and are concealed by scales deposited from the abdomen of the female. The eggs hatch after 3-4 days, larvae eating the remains of the egg shell before dispersing over the surface of the food source. There are six larval instars. Pupation occurs 14 days after hatching. Adults emerge 10 days later and usually die within 2 weeks.

2.1.3 Heliothis virescens Fabricius (Lepidoptera, Noctuidae)

Pupae reared on a semi-artificial diet were imported from Bioenvironmental Insect Control Laboratory, Stoneville, U.S.A. They were allowed to emerge and held at 25°C, 70-80% R.H., LD 12:12 and fed on a 5% (wt./vol.) honey solution.

2.2.1 Formamidine Insecticides

Chlordimeform (CDM)

Analytical grade chlordimeform base (99% pure), N'-(4-chloro-2-methyl)-N,N-dimethylformamidine (C8514) and its hydrochloride (CDM. HCl) and samples of the principle metabolites (Table 1, Fig. 2) were supplied by Ciba-Geigy Ltd., Basle, Switzerland.

2.2.2 Principle metabolites of CDM

(1) Demethylchlordimeform (DCDM)

N'-(4-chloro-2-methylphenyl)-N-methylformamidine (CGA 41 655).

(2) 4'chloro-o-formotoluidine

4-chloro-2-methyl-N-formylaniline (CGA 41 658)

(3) 4-chloro-2-methylaniline (CGA 41 657)

(4) 4-chloro-2-carboxy-N-formylaniline (CGA 20 958)

(5) 4-chloro-2-carboxyaniline (CGA 20 957)

2.2.3 Amitraz

Technical grade Amitraz (N,N-di(2,4,-xylyliminomethyl)-methylamine, externally stabilised by paraformaldehyde, was supplied by the Boots Co. Ltd., Nottingham.

2.2.4 Other Insecticides

Organochlorine: p,p-DDT

Analytical grade DDT (99% pure), 1,1,1,-trichloro-2,2-di(chlorophenyl)ethane was obtained from the National Physical Laboratory.

Carbamate:carbaryl

Analytical grade carbaryl, (99% pure), 1-napthyl methylcarbamate was supplied by Union Carbide, U.K., Ltd..

Pyrethroid:permethrin

Technical grade permethrin (cis 21%, Trans 73%), 3-phenoxybenzyl-(±)-cis, trans-3-(2,2,dichlorovinyl)-2,2, dimethylcyclopropanecarboxylate was supplied by Mitchell Cotts Chemicals.

2.2.5 Other Chemicals

The synergist, piperonyl butoxide (80% P.B., 20% other synergists) was obtained from Wellcome Research Laboratories, Berkhamsted.

All chemicals were of analytical grade and were obtained from Sigma (London) PLC or B.D.H. PLC unless otherwise stated. All radiochemicals were purchased from Amersham International PLC.

2.3.1 Liquid scintillation counting

Bray's scintillant (Bray, 1960) and p-terphenyl, (6g/dm^3) were used for aqueous and organic samples, respectively. Ten cm^3 of scintillant were added to low potassium glass vials or 5 cm^3 to polypropylene vial inserts.

Vials were allowed to stand for some hours in the dark before counting in a Beckman L250 liquid scintillation counter to a 2 Sigma error of 0.5%.

The results were accumulated on punched paper tape corrected for background and counting efficiency by the external standards-channels ratio method using a computer programme written in Fortran.

Quench calibration curves for each scintillant solution and for both types of vial were constructed using ^{14}C (1.115 x 10^6 dpm/g) and ^3H n-hexadecane (4.44 x 10^6 cpm/g, with allowance for decay). ^{14}C and ^3H samples were counted with an efficiency of about 95% and 25% respectively.

2.3.2 Double label counting (^3H and ^{14}C)

Tritium and ^{14}C can be counted simultaneously under optimum conditions for both compounds by differentiating between the energies of beta particles emmited by the two isotopes.

The pulse height analyser was set so that virtually all tritium was excluded from the higher energy ^{14}C window. The tritium channel contained a proportion of the count from the ^{14}C isotope, which increased with quenching. Three quench tables were constructed;

Table 1 = efficiency of counting ^{14}C in ^{3}H channel.

Table 2 = efficiency of counting ^{3}H in ^{3}H channel.

Table 3 = efficiency of counting ^{14}C in ^{14}C channel.

After subtracting the background for each channel the results were processed by digital computer using the following scheme where,

C_1 = CPM in ^{3}H channel from ^{14}C and ^{3}H.

C_2 = CPM in ^{3}H channel from ^{14}C only.

C_3 = CPM in ^{14}C channel from ^{14}C only.

C_4 = CPM in ^{3}H channel from ^{3}H only.

<u>^{14}C Activity</u>

$$\text{DPM } (^{14}C) = \frac{C3}{T3}$$

<u>^{3}H Activity</u>

The proportion of ^{14}C in ^{3}H channel was calculated by the expression

$$C2 = \text{DPM } (^{14}C) \times T1$$

This value was subtracted from ^{3}H activity (in CPM)

$$C4 = C_1 - C_2$$

and converted to DPM

$$\text{DPM } (^{3}H) = \frac{C4}{T2}$$

Chapter 3

TOXICOLOGICAL STUDIES

3.1 Introduction

Five methods have frequently been used to apply compounds to insects for evaluating toxicity. Dipping into solutions, direct spraying, contact, topical application and microinjection.

The insecticides were applied routinely using the last two methods as they are the most reproducible techniques. Microinjection involves piercing the cuticle and may stress the insect. Topical application is less disruptive but variation may occur in the rate of penetration through the cuticle.

3.2 Materials and Methods

3.2.1 Selection of insects: adults

Adults were sexed at the pupal stage and treated on the day after emergence. Moths were placed in individual 5 cm dia. Petri dishes and randomly assigned to test groups. After weighing, adults were treated with the appropriate concentration of insecticide or solvent only for the controls. To account for possible circadian rhythms in the susceptibility to insecticides, insects were treated in the first quarter of the light cycle. Treated insects held under the same conditions as the main culture (Section 2.1.1). Toxicity was assessed after 24, 48 and 60 h. noting the symptoms reported by Sawicki (1962) for houseflies, namely: (i) Incoordination - insects hyperactive but able to walk; (ii) knocked down - insects unable to walk but showed body movements; (iii) death - absence of movements even after tactile stimulation.

3.2.2 Selection of insects: larvae

Larvae were distinguished by the width of the head capsule (Appendix 2) since weights of different instars overlapped. After selecting the appropriate instar, healthy individuals were weighed and those falling into the following weight ranges were used: fourth instar, 60-90 mg; fifth instar, 200-250 mg and sixth instar, 670-800 mg.

3.2.3 Topical application of insecticide

A number of solvents were evaluated for use in topical application of insecticides. Ideally, the solvent should not be toxic and should spread evenly over the cuticle without persisting as this reduces penetration of the pesticide. In addition, if the solvent is too volatile, microlitre quantities cannot be applied accurately (Hadaway et al., 1976).

Acetone (b.p. 56°C) has been used extensively in toxicological studies with insecticides. However, it evaporates so rapidly there was no spreading, leaving a concentrated deposit. In contrast, preliminary experiments in this study showed ^{14}C hexadecane (b.p. 287°C) could be applied accurately in nanolitre volumes, but persisted for some hours (as shown by fluorescent dyes) reducing penetration and the toxicity of chlordimeform. Haddaway et al. (1976) reported di-isobutylketone (2,6,dimethylheptan-4-one) (b.p. 168°C) to be one of the most effective solvents for applying insecticides, but this proved toxic to S. littoralis. Methyl ethyl ketone (M.E.K, 2-butanone, b.p. 80°C) gave reproducible results with no toxic effects and was used routinely for all topical application. An exception was CDM.HCl which was not very soluble in M.E.K, and ethanol was used as the solvent.

Compounds were applied using an all glass syringe fitted with a 25 gauge needle, bent at 90°, and mounted on an Arnold microapplicator.

The insecticides were deposited on the dorsal surface of the adult thorax, or on the three thoracic segments of the larvae.

No anaesthesia was used during application of insecticide because, although carbon dioxide has been extensively used to immobilise test insects, the spiracles remain open which can enhance penetration. Haddaway et al. (1976) discovered that for a constant amount of insecticide, toxicity was enhanced by increasing the volume of solvent applied, possibly as a result of disrupting the cuticle. Therefore a standard volume of solvent was used (1 µl) except for application of formamidines to sixth instar larvae where 5 µl was required to apply the higher concentrations of insecticide.

Where mortality occurred in control insects, the assay was not included in estimating the median lethal dose.

Individual larvae were held in 5 cm dia. Petri dishes with artificial diet, supplied ad lib. under the same conditions as the adults. Toxicity was assessed as above.

3.2.4 Injection of insecticides

To reduce mortality due to nuclear polyhedrosis virus, all equipment was autoclaved before use and the Arnold microapplicator mounted in a microflow cabinet. The dorsal surface of the insect was sterilised with absolute ethanol. After injecting the test solution into the thorax with a 33 guage needle, the wound was sealed with beeswax. Using these precautions, no mortality was observed in the controls.

3.2.5 Incorporation of insecticides into artificial diet

CDM base was unsuitable being unstable when incorporated into the diet. CDM.HCl and amitraz were mixed with the diet at 40 °C and allowed to set. Weighed amounts of the diet were administered to fifth instar larvae.

3.2.6 Oral application of insecticides

Starved larvae were observed to drink small, 5 µ droplets of solutions containing phagostimulants such as sucrose or sinigrin. Fifth instar larvae were therefore starved for 4 h and placed in Petri dishes with 5 µl droplets of a 1% (wt/vol) solution of sinigrin on a hydrophobic surface (Parafilm). However, it was found that even low concentrations of formamidines were extremely repellent. Fifth instar larvae were therefore force-fed with 5 µl solutions containing various concentrations of CDM.HCl.

3.2.7 Application of synergist (Piperonyl butoxide)

Synergists have frequently been applied in the ratio of 10 parts synergist to 1 part insecticide (Casida, 1970). In the present study it was decided to use one concentration of synergist, with varying concentrations of formamidines. The concentration of synergist used was the greatest that could be applied without causing mortality in the controls.

Fourth instar larvae of S. littoralis were treated with 100 mg piperonyl butoxide (P.B.) in 1 µl M.E.K. To reduce possible interference in penetration of insecticide (Casida, 1970), the synergist was applied to the ventral surface of the thorax one hour before application of the formamidines to the dorsal side. To ensure there was always sufficient concentration of piperonyl butoxide to inhibit the action of microsomal mixed function oxidases, in preliminary bioassays larvae were placed on filter paper impregnated with synergist to continuously dose the test insect. Adult H. virescens were similarly treated with 2.5 µg of P.B. in 1 µl of M.E.K. one hour before applying the formamidines.

3.2.8 Analysis of mortality data

Probit regression lines were fitted to bioassay results according to the maximum likelihood method of Finney (1952) using a computer programme (Davies, 1971) modified by A.R. Ludlow.

The dose expected to produce a response in fifty percent of the population (LD_{50}) was calculated, together with the slope and intercept of the regression line, the variance of the slope and a chi squared value to test for homogeneity of the data. The last value was compared with tables of this statistic for n-2 degrees of freedom (where n is the number of experiments). Heterogeneity was indicated if this value was greater than 2 at the 5% level. An adjustment factor was calculated from 2/degrees of freedom and the variance multiplied by this result; 95% fiducial limits for the probit regression line were calculated from the variance.

3.3 Results

3.3.1 Symptoms of formamidine toxicity in adults

Table 7 summarises the symptoms of formamidine poisoning in adults. At high concentrations, almost immediate symptoms of hyperactivity were observed. Moths flew during the day, when normally they were quiescent, becoming uncoordinated before falling to the ground. The wings beat violently for many hours. This was followed by a comatose phase in which air sacs expanded distending the abdomen. The proboscis, legs and antennae were periodically extended. In males, claspers were opened (Plate 3) while in females many scales were shed from the abdomen, which are normally used to conceal eggs (Plate 4). Few eggs were laid at high concentrations. Sometimes individuals failed to uncouple after mating (Plate 5). The comatose phase may last for many hours before death. Similar symptoms were seen at lower doses although they were less severe.

The slow onset of symptoms and length of time required to kill insects are typical of formamidines. Representatives of other classes of insecticides produced immediate symptoms such as hyperactivity (sevin) or knockdown (DDT). All types caused adults to ingest air and this is probably a general response to stress, but no group caused prolonged and violent wing beating.

3.3.2 Symptoms of formamidine toxicity in larvae

The symptoms of poisoning after injection, ingestion or topical application were similar. Tremors were observed in the head and mouth parts and larvae became hyperactive and uncoordinated. After some hours they were unable to crawl despite vigorous movements of the prolegs. During the comatose phase, larvae appeared dead but responded to tactile stimulation. The median lethal dose required to kill different stages on S. littoralis and H. virescens are given in Tables 11-17. The LD_{50} is frequently estimated after 24 h but because of the characteristically slow onset of symptoms with formamidines, mortality was calculated after 48 and 60 h.

3.3.3. Effect of formamidines on feeding

Topical application of 1 and 5 µg of CDM to fifth instar larvae significantly reduced feeding after 24 and 48 h as measured by mean increase in body weight (Table 8).

CDM.HCl significantly inhibited feeding after 24 h when incorporated into the diet at the rate of 1000 to 10,000 $\mu g/cm^3$. Concentrations as low as 100 $\mu m/cm^3$ were effective after 48 H (Table 9).

In both experiments, larvae were kept until adults emerged. In many individuals metamorphosis was only partly complete and pupae were deformed. Using formation of normal pupae as the criteria, the

LC_{50} was 300 $\mu g/cm^3$. Many adults failed to emerge from apparently normal pupae. Generally, those which did emerge were smaller than the controls and a number failed to expand their wings. Using emergence of normal moths as the criteria, the LC_{50} was 94 $\mu g/cm^3$.

In addition, at concentrations above 1000 $\mu g/cm^3$, adults emerged with symptoms of poisoning such as hyperactivity and shedding of abdominal scales, observed when moths were treated topically with formamidines.

3.3.4 Effects of formamidines on fecundity

Topical application of 10 and 1µg of CDM base of female moths reduced the mean number and weight of eggs laid per gram body weight. The number of egg masses was actually increased at 0.1µg CDM but there were fewer eggs per batch (Table 10).

Treating the male had no effect on the number of eggs laid by untreated females.

PLATE 1. Sixth instar S. littoralis larvae.

Scale bar = 1 cm.

PLATE 2. Adult S. littoralis.

Scale bar = 1 cm.

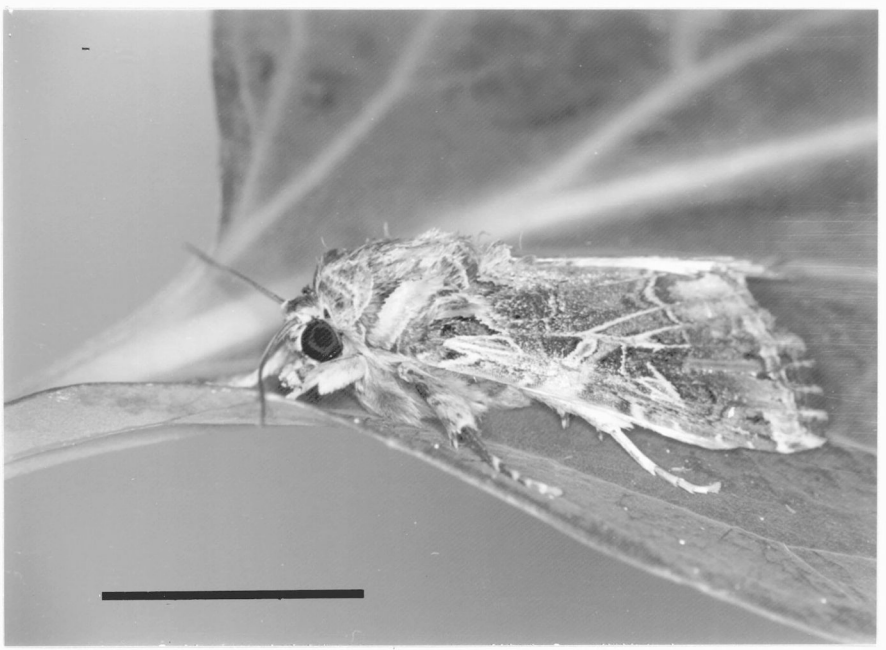


PLATE 3. Adult male 24 h after topical application of an LD_{50} of CDM.HCl.

Scale bar = 1 cm.

PLATE 4. Adult female 24 h after topical application of an LD_{50} of CDM.

Note shed abdominal scales.

Scale bar = 1 cm.

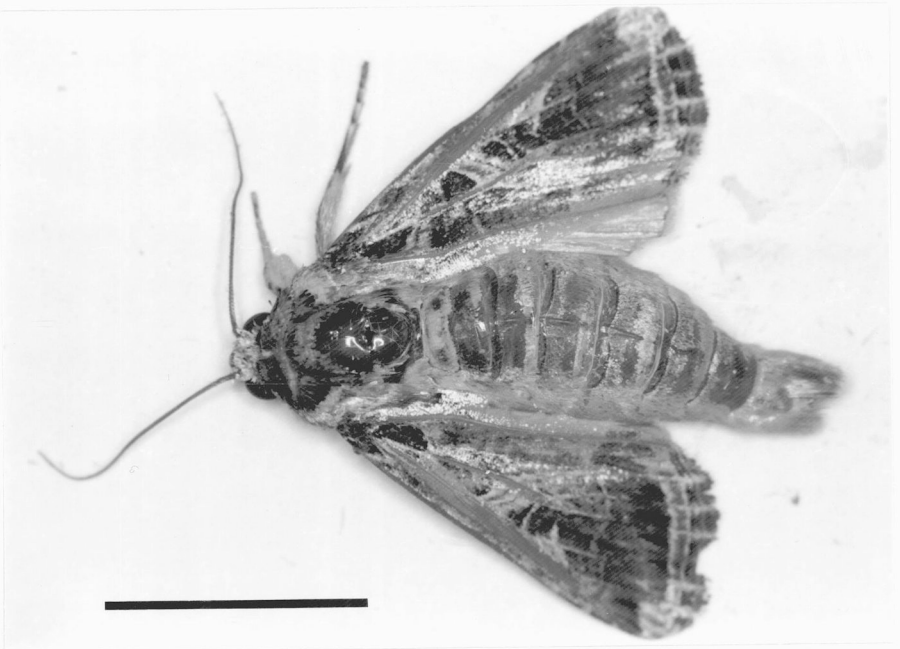


Plate 5. Failure of adults to uncouple after mating following topical application of CDM.HCl.
Scale bar = 1 cm.

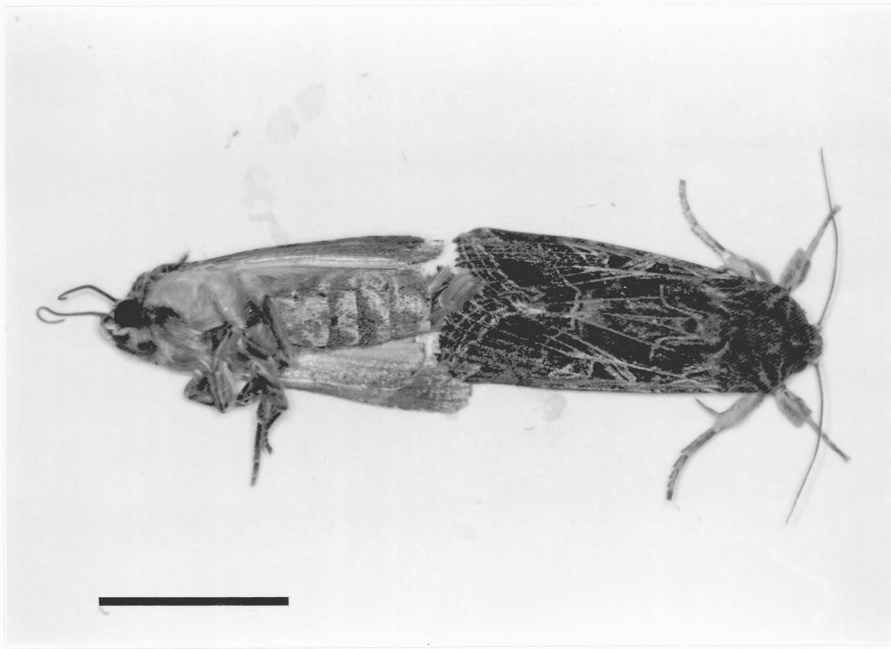


Table 7. Symptoms of poisoning in S. littoralis adults following topical application of CDM.

µg/moth	
100	ALMOST IMMEDIATE SYMPTOMS: HYPER-ACTIVITY, SCALES SHED FROM ABDOMEN AND WINGS; COMATOSE PHASE, SWOLLEN ABDOMEN, EXTENDED GENITAL PLATES.
10	ALTERATION OF EGG LAYING BEHAVIOUR ABDOMINAL SCALES SHED, SOME EGGS. HYPERACTIVITY AND COMATOSE PHASE SOME TIME AFTER TREATMENT.
1	REDUCTION IN NUMBER OF EGG MASSES FEWER EGGS PER MASS SHEDDING OF ABDOMINAL SCALES
.1	EGG MASSES ALMOST NORMAL MANY SHED ABDOMINAL SCALES, SOME HYPERACTIVITY AFTER 48 HOURS
.01	NORMAL EGG MASSES. LITTLE OR NO EFFECT ON BEHAVIOUR

Table 8. Effect of topically applied CDM on larval feeding.

	Concentration of CDM (µg/larvae)			
	Control	0.5	1	5
Mean weight of larvae (mg) ± S.E.M. (n = 10)	222.3±4.0	222.3±4.0	222.0±4.0	222.9±4.2
Mean increase in weight (mg) ± S.E.M. 24 h after treatment	586.9±42.1	497.5±46.5 t = 1.425+	443.7±45.3 t = 2.310*	457.1±43.0 t = 2.150*
Mean increase in weight (mg) ± S.E.M. 48 h after treatment	958.8±41.8	850.0±49.7 t = 1.844+	694.8±71.9 t = 3.356*	745.6±32.9 t = 4.665**

* Significant at 5% level.

** Significant at 1% level.

\+ Not significant. (Related t-test)

Table 9. Effect of CDM.HCl incorporated in the diet on larval feeding.

	Concentration of CDM.HCl in diet ($\mu g/cm^3$)						
	Control	10,000	5,000	2,500	1,000	500	100
Mean weight of larvae (mg)±S.E.M. (n = 10)	219.4± 1.8	219.4± 1.8	219.6± 1.8	219.6 1.8	221.5± 2.1	219.9± 1.9	220.3± 2.0
Mean increase in weight after 24 h	433.6±20.9	239.8±24.1 t=5.549**	215.5±14.1 t=7.894**	272.7±19.5 t=4.987**	317.7±27.9 t=3.033*	379.8±24.0 t=1.546+	409.1±26.6 t=0.661+
Mean increase in weight after 48 h	764.7±19.6	278.2±35.7 t=11.942**	283.1±42.1 t=10.377**	477.6±38.1 t=4.417**	430.4±49.8 t=6.265**	398.4±28.9 t=10.505**	574.2±65.0 t=2.802*

* Significant at 5% level.

** Significant at 1% level.

\+ Not significant. (Related t-test).

Table 10. Effect of CDM topically applied to S. littoralis moths on Fecundity.

	Concentration of CDM (µg/moth)			
	Control	10	1	0.1
Mean no. of eggs/g of female	7715	882	804	7407
Mean wt (mg) of eggs/g of female	399.4	53.0	48.9	430.2
Mean no. of eggs/ female	1747	190	168	1728
Mean wt (mg) of eggs/female	90.3	11.4	10.2	96.0
Mean no. of eggs/ batch	416	190	153	303
Mean wt (mg) of eggs/batch	22.0	11.4	9.3	16.8

TABLE 11. Toxicity of formamidines to adult S. littoralis at 48 and 60 hours following topical application.

		48 Hours			60 Hours		
Sex/ Formamidine	A:µg/g body wt. B:µg/insect	LD_{50}	95% Fiducial Limits	Slope	LD_{50}	95% Fiducial Limits	Slope
Female	A	216.3	147.9- 316.3	1.044	25.9	9.2- 73.5	0.8810
CDM	B	37.1	11.2- 123.1	1.046	4.5	1.6- 12.8	0.8735
Male	A	150.6	49.6- 457.8	0.9840	16.3	7.6- 34.9	1.819
CDM	B	19.3	6.2- 60.7	0.9325	2.2	1.0- 4.8	1.779
Female	A	1.4	0.65- 3.2	1.745	0.33	0.13- 0.81	1.370
CDM.HCl	B	0.27	0.12- 0.59	1.809	0.062	0.03- 0.15	1.385
Male	A	1.9	0.83- 4.3	1.744	0.18	0.07- 0.47	1.246
CDM.HCl	B	0.27	0.12- 0.59	1.809	0.026	0.01- 0.07	1.245
Male	A	3307.0	2387.6-4571.3	2.855	810.6	502.6-1308.6	1.808
Amitraz	B	396.2	69.5-2258.2	1.775	117.6	73.6- 189.2	1.825

TABLE 12. Toxicity of formamidines to S. littoralis larvae 48 and 68 hours following topical application, injection or ingestion.

		48 Hours			60 Hours		
Instar/ Formamidine	A:µg/g body wt. B:µg/insect	LD_{50}	95% Fiducial Limits	Slope	LD_{50}	95% Fiducial Limits	Slope
TOPICAL APPLICATION							
6th	A	546.6	286.1-1041.3	1.865	101.0	51.0-199.6	2.655
CDM	B	348.7	192.2- 634.3	1.997	26.8	10.0- 71.6	1.162
6th	A	625.4	520.4- 752.6	3.732	625.4	520.4-752.6	3.732
CDM.HCl	B	428.7	357.8- 514.4	3.809	428.7	357.8-514.4	3.809
5th	A	35.8	19.1- 61.2	1.662	35.8	19.1- 61.2	1.662
CDM	B	8.2	4.6- 14.6	1.667	8.2	4.6- 14.6	1.667
5th	A	515.0	592.4- 15.4	0.401	515.0	592.4-159.4	0.341
CDM.HCl	B	113.4	274.7- 15.1	0.3140	113.4	274.7- 15.1	0.314
4th	A	32.7	13.3- 80.4	1.442	32.7	13.3- 80.4	1.442
CDM	B	2.3	0.93- 5.5	1.449	2.3	0.93- 5.5	1.449
4th	A	419.5	325.4- 765.9	0.8723	419.5	325.4-765.9	0.872
CDM.HCl	B	27.6	7.3- 103.8	0.8942	27.6	7.3-103.8	0.894
4th	A	-	-	-	15430.0	9272.7-25632.7	5.146
Amitraz	B	-	-	-	1963.0	640.0- 1841.3	4.602
INJECTION							
6th	A	918.5	244.8-3760.2	0.555	349.0	240.9- 504.7	0.719
CDM.HCl	B	426.6	233.2- 782.0				
INGESTION							
5th	A	642.6	379.7-1085.0	1.558	317.9	151.9- 665.9	1.226
CDM.HCl	B	126.9	76.2- 211.2	1.608	63.2	25.0- 160.3	1.244
TOPICAL APPLICATION (Susceptible strain)							
4th	A	0.73	0.45- 1.2	1.546	0.55	0.34- 0.88	2.017
CDM.HCl	B	0.49	0.37- 0.65	2.515	0.41	0.31- 0.56	3.443

TABLE 13. Toxicity of formamidines and synergist to Heliothis virescens at 48 and 60 hours following topical application.

		48 Hours			60 Hours		
Formamidine	A:µg/g body wt. B:µg/insect	LD_{50}	95% Fiducial Limits	Slope	LD_{50}	95% Fiducial Limits	Slope
CDM.HCl	A	13.5	7.1 - 25.8	1.265	4.6	3.2 - 6.7	3.027
	B	1.7	0.96- 3.18	1.362	0.64	0.45 - 0.91	3.154
CDM.HCl+ P.B.	A	5.4	2.3 - 12.8	0.8222	0.91	0.63 - 1.3	2.636
	B	0.73	0.31- 1.7	0.8696	0.085	0.044- 0.17	1.790
DCDM	A	107.1	58.0 - 197.9	1.650	81.3	56.9 - 116.2	2.474
	B	12.8	7.7 - 21.4	1.890	12.2	8.6 - 17.1	2.580
DCDM+ P.B.	A	12.4	8.7 - 17.6	3.208	1.1	0.68 - 1.6	2.350
	B	2.4	1.5 - 3.8	2.629	0.42	0.68 - 2.6	2.640
Amitraz	A	5341.0	4455.5 -6413.7	3.524	3490.0	2996.9 -4067.5	4.860
	B	886.1	725.3 -1080.2	3.153	562.0	475.4 - 665.2	4.338
Amitraz P.B.	A	463.5	238.0- 902.5	1.079	127.0	68.9- 234.6	1.724
	B	71.7	37.6- 137.2	1.130	20.5	10.9- 38.6	1.681

TABLE 14. Comparison between injection and topical application of formamidines to H. virescens at 48 and 60 hours.

Formamidine	A: µg/g body wt. B: µg/insect	48 Hours LD_{50}	48 Hours 95% Fiducial Limits	48 Hours Slope	60 Hours LD_{50}	60 Hours 95% Fiducial Limits	60 Hours Slope
CDM.HCl	A	13.5	7.1 - 25.3	1.265	4.6	2.2 - 6.7	3.027
(Topical)	B	1.74	0.96- 3.18	1.362	0.64	0.45- 0.91	3.154
CDM.HCl	A	18.8	9.4 - 37.7	1.281	5.4	2.7 - 10.8	1.169
(Injection)	B	2.7	1.4 - 5.48	1.272	0.76	1.53- 0.38	1.173
CDM	A	1263.0	263.6 -6036.9	0.677	59.6	11.8 -301.8	1.065
(Topical)	B	142.0	30.9 - 653.2	0.700	29.7	11.4 - 37.3	1.795
CDM	A	634.0	257.4 -1624.2	0.784	297.8	130.5 -679.9	0.946
(Injection)	B	98.5	38.5 - 251.4	0.9780	46.9	20.3 -109.0	0.927

TABLE 15. Comparison between the toxicity of formamidines and other types of insecticide topically applied to fourth instar S. littoralis.

Insecticide	A: µg/g wet wt. B: µg/insect	48 Hours LD_{50}	48 Hours 95% Fiducial Limits	48 Hours Slope	60 Hours LD_{50}	60 Hours 95% Fiducial Limits	60 Hours Slope
CDM	A	32.7	13.3 - 80.4	1.442	32.7	13.3 - 80.4	1.442
	B	2.3	0.93 - 5.5	1.449	2.3	0.93- 5.5	1.449
CDM.HCl	A	419.5	325.4 - 765.9	0.8723	419.5	325.4 - 765.9	1.883
	B	27.6	7.3 - 103.8	0.8942	27.6	7.3 - 103.8	0.894
DDT	A	2393.0	1115.0 -5136.8	0.6067	76.5	23.6 - 247.1	1.883
	B	76.5	23.6 - 247.1	0.6762	198.3	44.5 - 2974.6	0.671
Carbaryl	A	3673.0	3232.0 -4173.0	0.6662	1557.0	84.1 - 1049.0	0.572
	B	296.1	84.1 -1049.0	0.7190	131.0	41.2 - 415.9	0.631
Permethrin	A	0.54	0.2 - 0.83	2.196	0.41	0.23- 0.70	1.449
	B	0.033	0.075- 0.15	1.487	0.045	0.03- 0.07	2.205
Amitraz	A	-	-	-	15430.0	9272.7 -25632.7	5.146
	B		-	-	1063.0	640.0 - 1841.2	4.602

TABLE 16. Toxicity of chlordimeform to adult S. littoralis 48 and 60 hours following topical application, in three different solvents.

Formamidine/ solvent	A: µg/g wet wt. B: µg/insect	48 Hours LD_{50}	48 Hours 95% Fiducial Limits	48 Hours Slope	60 Hours LD_{50}	60 Hours 95% Fiducial Limits	60 Hours Slope
Females							
CDM Acetone	A	28.1	11.2- 70.3	1.417	1.17	0.42 - 3.2	1.140
	B	2.6	1.0- 6.9	1.175	0.22	0.081- 0.59	1.161
CDM/HEXA-	A	256.7	77.3-851.0	1.545	26.9	8.1 - 89.8	1.523
DECANE	B	48.7	14.3-166.0	1.504	4.9	1.5 - 16.5	1.507
CDM/M.E.K.	A	216.3	147.9-316.3	1.044	25.9	9.2 - 73.5	0.8810
	B	37.1	11.2-123.1	1.046	4.5	1.6 - 12.8	0.8735
Males							
CDM/Acetone	A	43.9	17.7-109.2	1.399	2.3	0.7 - 7.6	2.237
	B	6.0	2.5- 14.7	1.425	0.32	0.1 - 1.1	2.198
CDM/HEXA-	A	30.2	8.8-103.8	1.489	19.5	6.2 - 60.3	1.783
DECANE	B	4.9	1.9- 16.5	1.507	3.2	1.0 - 9.6	1.809
CDM/M.E.K.	A	86.1	17.8-416.8	0.5953	16.3	7.6 - 34.9	1.819
	B	11.6	2.4- 55.1	0.6004	2.2	1.0 - 4.8	1.774

TABLE 17. Toxicity of formamidines and synergists to fourth instar S. littoralis larvae.

		48 Hours			60 Hours		
Formamidine	A: µg/g wet wt. B: µg/insect	LD_{50}	95% Fiducial Limits	Slope	LD_{50}	95% Fiducial Limits	Slope
CDM	A	32.7	13.3 - 80.4	1.442	32.7	13.3 - 80.4	1.442
	B	2.3	0.93- 5.5	1.449	2.3	0.93- 5.5	1.449
CDM + P.B.	A	127.8	96.2 - 169.5	2.686	78.6	55.4 - 111.3	2.397
	B	7.1	5.0 - 10.1	2.203	3.8	2.5 - 5.9	1.987
DCDM	A	2200.0	1653.4 -2921.6	1.914	2200.0	1653.4 -2921.6	1.914
	B	147.0	60.1 - 359.2	1.910	147.0	60.1 - 359.2	1.910
DCDM + P.B.	A	2362.0	1449.4 -3715.9	2.526	2293.0	1766.3 -2971.1	2.138
	B	144.3	96.6 - 215.2	2.861	140.2	89.8 - 219.1	2.420

3.4 Discussion

3.4.1 Toxicity of formamidines to Adults and Larvae

The toxicity of the formamidines to S. littoralis and H. virescens is given in Tables 11-17. Males of most insect species are usually more susceptible to contact insecticides than the females (Busvine, 1971). This was found following treatment with CDM but not CDM.HCl (Table 11).

There was a dramatic and unexpected difference of two orders of magnitude between the toxicity of the base and salt to both males and females. Such a difference has not been previously reported. The volatility of the base may be important in this context, leading to loss of insecticide from the cuticle. Other factors, such as persistance of the solvent might also be involved. This difference between CDM and CDM.HCl was also found using H. virescens adults (Table 14) where the salt was nearly 100 times more toxic to females. Loss of the base from the cuticle only partially explained this difference. When the two compounds were injected into H. virescens, thereby eliminating differences in penetration and volatility, the salt remained more toxic than the base (Table 14). Both species of moth were much less susceptible to amitraz than CDM (Tables 11 and 13).

Table 18 compares the toxicity of formamidines to selected organisms. In the present study, extrinsic factors such as temperature, humidity and diet which are known to effect the toxicity of insecticides were controlled. In most reports however, the bioassay conditions were not described, which complicates comparison between insect species.

The LD_{50}^{48}(µg/g body weight) for CDM.HCl of 1.4 and 216 for CDM (Table 11) compares with a value of 7.5 for CDM reported by Salvisberg et al. (1980a) for adult S. littoralis and of 3.32 for CDM in adult M. sexta (Lund et al., 1979b). These values are much lower than, for example, Musca domestica an insect not sensitive to CDM; an

LD_{50}^{48} of 2083 was recalculated from the data for Knowles and Shrivastava (1976). The authors demonstrated CDM was rapidly broken down in houseflies and differential metabolism was the most important factor in selective toxicity of formamidines.

The concentration of CDM and CDM.HCl in µg/g body weight required to kill S. littoralis larvae increased with age (Table 12). This reflects a general trend in other species. Tolerance increases with larval growth, although there are exceptions. For example, some first instar larvae are exceptionally tolerant to certain insecticides (Busvine, 1971). Increased cuticle thickness, metabolism or storage in fat body have all been suggested to explain this phenomenon.

Differences in the toxicity of the salt and base were also observed in the larvae, but the results were reversed, the base being more toxic (Table 12).

CDM is known to be directly toxic to eggs and neonate larvae (Salvisberg et al., 1980a), but toxicity decreases rapidly from the fourth to sixth instar. The final instar was very tolerant. Some sensitivity is regained in the adult (Tables 11 and 12).

Sixth instar larvae were slightly less sensitive to CDM.HCl when injected (LD_{50}^{48} = 918) compared to topical application (LD_{50}^{48} = 625). Similarly, topical application was more effective than ingestion in killing fifth instars (Table 12).

Amitraz was not toxic to sixth or fifth instar S. littoralis and the median lethal dose could only be estimated in fourth instars 60 h following application. Only insect eggs, neonate larvae and adults were killed by this formamidine (Tables 11 and 18).

Fourth instar larvae of the Cairo strain of S. littoralis tolerated about 500 times more CDM.HCl than the strain susceptible to conventional insecticides (Table 12). Table 15 emphasises the tolerance of the Cairo strain to representatives of carbamate and organochloride

Table 18. Toxicity of formamidines to selected organisms.

Organism	Method of application	Toxicity		Reference
Lepidoptera				
Spodoptera littoralis		µg/g body wt.		
Eggs	Adsorption	LD_{50}^{-}= 4	CDM	Salvisberg et al. (1980a)
3rd Instar	Oral	LD_{50}^{-}= >200	CDM	El-Guindy et al. (1981)
4th Instar (35-40mg)	Topical	LD_{50}^{48}= 516.4	CDM	Salvisberg et al. (1980a)
Adults	Topical	LD_{50}^{24}= 40	CDM	"
		LD_{50}^{48}= 7.5	CDM	"
Spodoptera endania		µg/insect		
Adult	Topical	0.15	CDM	Gemrich et al. (1976)
Trichoplusia ni		µg/g body wt.		
3rd Instar (20mg)	Topical	LD_{50}^{48}= 750	CDM	Crecelius &
5th Instar (130mg)	"	LD_{50}^{48}= 1346	CDM	Knowles (1976)
3rd Instar (20mg)	"	LD_{50}^{48}= 3500	DCDM	
5th Instar (130mg)	"	LD_{50}^{48}= >4615	DCDM	
Manduca sexta		µg/g body wt.		
Eggs	Dipping	LD_{50}^{-}= 0.1%	CDM	Lund et al.
2nd Instar	Injection	LD_{50}^{48}= 700	CDM	(1979b)
5th Instar	"	LD_{50}^{48}= 406	CDM	
Adult	"	LD_{50}^{48}= 3.32	CDM	
Heliothis armigera		conc. of solution		
Eggs	Dipping	LC_{50}^{60}= 0.055%	CDM.HCl	Clift (1979)
2nd Instar	"	LC_{50}^{60}= 0.144%	CDM.HCl	
Eggs	"	LC_{50}^{60}= 0.865%	Amitraz	
2nd Instar	"	LC_{50}^{60}= >1.0%	Amitraz	

Organism	Method of application	Toxicity		Reference
Diptera		µg/g		
Adults	Topical	LD_{50}^{48}= 2083	CDM	Knowles & Shrivastava (1973)
Chilo suppressalis		µg/insect		
Adults	Topical	LC_{50}^{72}= >2000µg/cm³	CDM	Hirano et al. (1972)
Blattaria				
Blatta germanica		µg/g		
Adult	Injection	LD_{50}^{-}= 650	CDM	Hollingsworth (1976)
		LD_{50}^{-}= 555	CDM	
Periplaneta americana				
Adult	Injection	LD_{50}= 500	CDM	Beeman & Matsumura (1974)
Vertebrates				
Rat	Oral	LD_{50}^{-}= 250	CDM	Knowles (1976)
	Oral	LD_{50}^{-}= 335	CDM.HCl	"
	Dermal	LD_{50}^{-}= 4060	CDM.HCl	"
Rabbit	Oral	LD_{50}^{-}= 625	CDM	"
Mouse	Oral	LD_{50}^{-}= 285	CDM	Hollingsworth (1976)
	Injection	LD_{50}^{-}= 104	CDM	"
	Oral	LD_{50}^{-}= 230	DCDM	"
	Injection	LD_{50}^{-}= 93	DCDM	"

insecticides which have been extensively used to control S. littoralis. Median lethal doses were in the milligram range.

Three solvents were evaluated for topical application of insecticides (Table 14). CDM in acetone gave the highest mortality but was not suitable for microapplication. The rate of flow of this solvent from the needle was too rapid for accurate application and the high rate of evaporation increased the CDM concentration. The apparent higher mortality may have been caused by applying higher doses than expected. Despite these difficulties, acetone is still the preferred solvent for topical application bioassays (Busvine, 1971).

Both M.E.K. and hexadecane could be applied accurately. They spread over the cuticle persisting for some time and gave similar toxicological results. However, hexadecane was more toxic than M.E.K. and the latter solvent was used routinely.

3.4.2. Modification of formamidine toxicity by a synergist

DCDM was the only metabolite of CDM to show any biological activity (Tables 13 and 17). The four other metabolites (Section 2.2.2) were not toxic. DCDM was much less toxic than the parent compound (Table 17) in fourth instar S. littoralis larvae.

Piperonyl butoxide did not increase the toxicity of DCDM and only slightly synergised CDM. A similar relationship was found by Creceluis and Knowles (1976) in Tricoplusia ni larvae; DCDM was much less toxic than CDM, although piperonyl butoxide slightly enhanced DCDM toxicity.

In adult H. virescens the LD_{50}^{48} for DCDM was 107 µg/g body weight compared to 13.5 for CDM. Piperonyl butoxide enhanced the toxicity of both compounds. Amitraz was much less toxic than CDM but considerable synergism occurred with piperonyl butoxide (Table 13).

In all organisms that have been examined, CDM is metabolised to DCDM by microsonal mixed function oxidase (Fig. 3). Piperonyl butoxide inhibits this metabolism (Knowles, 1976). Inhibition of oxidative metabolism can enhance toxicity if the parent compound is active. Conversely, antagonism occurs when an insecticide must be oxidised to become active. For example, treatment of ticks with piperonyl butoxide reduced CDM toxicity but in this instance enhanced DCDM activity. DCDM was found to be 700 fold more effective than CDM in stimulating tick detachment (Knowles and Roulston, 1972). These authors provided further evidence supporting the idea that N-monomethylformamidines were the toxicants and not the parent compounds such as CDM (Knowles & Roulston, 1973). Metabolism of CDM may explain the delay of several hours in lighting the lantern in P. pyralis whereas DCDM produced an immediate response (Section 1.5).

Ticks and fireflies may be an exception. In houseflies (Knowles & Shrivastava, 1975), mites (Kuwahara, 1977), cockroaches (Hollingsworth, 1976), Lepidoptera (Crecelius & Knowles, 1976) and Tables 13 and 17, CDM is equally or more toxic than DCDM.

Amitraz is rapidly hydrolysed to the corresponding N-demethyl analogue, N'(2,4,-xylyl)-N-methylformamidine patented as BTS-27271 and 2,4,formoxylidide (Benezet et al., 1978).

The former compound differs from DCDM only in replacement of a chlorine by a methyl group at the 4 position on the ring. Hydrolysis would not be affected by synergists but compounds such as piperonyl butoxide would inhibit the further oxidative degradation of N-dimethyl products. Hence the synergism with amitraz (and DCDM) in adults of both species.

Similar results were found in ticks. CDM, DCDM, amitraz and BTS-27271 were almost equally toxic but synergism with piperonyl

butoxide occured only with the latter compounds; CDM toxicity was antagonised. BTS-27271 may be responsible for most, if not all, the toxicity associated with the parent compound, amitraz.

In the locust, octopamine receptors have been assigned to three classes (octopamine$_1$, octopamine$_{2A}$, octopamine$_{2B}$) on the basis of their interaction with agonists and antagonists (Evans, 1981). This raises the intriguing possibility that CDM and DCDM may interact with different octopamine receptors and the distribution of these classes varies between species. Currently, there is no information from the locust to support this hypothesis (Evans, 1982).

In the broken cell preparation from P. pyralis (Section 1.5), in contrast to DCDM, CDM was only a weak octopamine agonist (Nathason & Hunnicutt, 1981). Octopamine receptors have not yet been investigated in ticks but it is possible they are similar to the firefly, and only interact with demethylated formamidines, such as DCDM and BTS-27271. The parent compounds were equally toxic because CDM was oxidised to DCDM and amitraz was hydrolysed to BTS-27271.

In other organisms, both classes of receptor may be present in Lepidopterans. CDM toxicity may result principally by interaction with receptors responding to parent formamidines and to a lesser extent to demethylated metabolites. With amitraz only the demethylated metabolite may react to produce toxicity, and this could explain the insensitivity of S. littoralis larvae and adults of both species.

3.4.3 Effect of formamidines on feeding

Before feeding begins in Lepidopterous larvae, behavioural patterns bring the head and mouthparts into a suitable position to monitor the quality of the food by sensilla located on the mouthparts (Schoonhoven, 1973). If the chemoreceptors indicate the food is acceptable, an initial bite is taken. Olfaction plays a major role in

feeding. Larvae possess sensory neurones specific for different types of phagostimulants (for example, water, glucose and sinigrin) and feeding deterrents, mainly secondary plant metabolites, such as amines and glycosides (Schoonhoven, 1973). Phagostimulants are essential to initiate and maintain feeding, but the balance between phagostimulants and deterrents determines the palatability of the food.

The artificial diet used for S. littoralis contains many phagostimulants such as sucrose and sinigrin to initiate and maintain feeding. Thirsty larvae readily drink small droplets containing sucrose and sinigrin. When CDM.HCl was included, larvae were observed to make initial contact with sensilla located on the mouthparts then rapidly moved away from the droplet. Further contact was avoided. In this case, the formamidine was acting as a repellent.

When CDM.HCl was incorporated into the artificial diet, larvae were not repelled immediately but fed initially on the treated food. Although reduction in feeding is probably a general effect of any toxic compounds, at sub-lethal doses CDM.HCl acts as an antifeedant. The mechanism is unknown. Formamidines may mimic the action of feeding deterrents on specific sensilla. S. littoralis is polyphagous and has been reported to feed on over 200 host plants suggesting the number of feeding deterents to this moth are very few.

When formamidines are applied topically to larvae, they cannot interact with cuticle chemoreceptors, but may effect processes which regulate meal size.

In insects, meal size is regulated to prevent physical damage by overeating. Feeding proceeds until inhibited by stretch receptors overriding chemosensory information (Barton Browne, 1975). In locusts, sectioning of the posterior pharyngeal nerves at the front of the foregut causes hyperphagia suggesting these nerves conduct the signal for cessation of feeding (Bernays & Chapman, 1973). Stretching of the

crop releases one or more hormones from the storage lobe of the corpora cardiaca. For example, the diuretic hormone decreases haemolymph volume. Haemolymph composition changes with removal of water into the gut, raising the osmotic pressure, and the concentrations of ions, amino acids, carbohydrates and lipids (Barton Browne, 1975). Formamidines could interfere with one or more of these regulatory systems, such as the formamidine induced elevation of haemolymph carbohydrate and lipid, so reducing feeding. Appetite loss would contribute to toxicity particularly in rapidly developing neonate larvae.

3.4.4 Effect of formamidines on fecundity

Reduction in fecundity has frequently been observed in insects and acarines treated with formamidines but how this occurs has not been explained (Table 4).

In most insects, mating stimulates oogenesis and provokes an abrupt change in behaviour of the female. In moths, the presence of sperm in the female spermatheca is the trigger. Mating with castrated males involving the passage of sterile spermatophores did not cause this switch in behaviour (Truman & Riddiford, 1971). The moth corpora cardiaca has a central role in controlling reproduction. The presence of sperm in the female spermatheca causes a reflex mechanism in the brain to release a chemical message from the intrinsic cells of the corpora cardiaca leading to an increased oviposition rate, switching from the virgin to the mated condition. Neural connections between brain and corpora cardiaca must be intact for this to occur (Truman & Riddiford, 1971).

Prostaglandin synthetase is present in the male house cricket, Acheta domestica but cannot be detected in the female reproductive tract until after mating. Similarly, a PGE_2-like compound was present in the mated female tract but not virgin females. Prostaglandin

synthetase inhibitors suppressed oviposition when a treated female was mated with an untreated male. The reciprocal cross had no effect on egg laying, suggesting insufficient inhibitor entered the reproductive tract via the spermatophore to inhibit reproduction (Destephano & Brady, 1977). These authors concluded post-copulatory biosynthesis was probably responsible for triggering oviposition. If a similar mechanism operated in moths, prostaglandin synthetase could be the factor leading to the formation of a spermathecal hormone (such as prostaglandin). This in turn might act directly on the neurosecretory centres to trigger the release of factors from the corpora cardiaca which switch females from a virgin to a reproductive state. A similar result was obtained with CDM (Table 10) to prostaglandin synthetase inhibitors, treatment of female but not male S. littoralis reducing fecundity. Formamidines are known to inhibit prostaglandin synthetase (Section 1.5) but whether these compounds reduce fecundity by inhibition of this enzyme or by interfering with other components involved in mating, has not been investigated.

Chapter 4

HISTOCHEMICAL LOCALIZATION OF BIOGENIC AMINES

4.1 Introduction

Incubation of nervous tissue with saline containing the dye Neutral red results in the selective staining of aminergic cells. Stuart et al., (1974) found a good correlation between Neutral red staining and the formaldehyde induced fluorescence (FIF) method for cells containing 5-hydroxytryptamine and dopamine. Since octopaminergic cells stain with the former but do not react with formaldehyde in the latter method, a combination of the two techniques can be used in an attempt to localise cells containing octopamine.

The FIF (Falck-Hillarp) method is based on the condensation of primary (noradrenaline, dopamine and 5-hydroxytryptamine) and secondary monoamines (adrenaline) with formaldehyde vapour when the amines are enclosed in a dry protein matrix. Under these conditions, intensely fluorescent compounds are formed. In the initial reaction leading to fluorophore formation a high electron density is required at the point of ring closure; the -6-position of phenylethylamines and -2-position of indolethylamines. Tyramine and octopamine lack substitutes at the 3 position of the ring and are non-fluorogenic.

It is essential to perform all procedures under completely dry conditions. Adequate freeze drying of the tissue is probably the most critical stage. The technique is highly sensitive and specific, detecting as little as 5×10^{-6} pmoles of amine (Falck & Owman, 1965).

4.2 Materials and methods

4.2.1 Neutral red method

Nervous tissue was dissected from sixth instar and adult insects and placed in a freshly prepared, filtered (Whatman No.1) solution of Neutral red (0.001%, wt/vol) in S. littoralis saline (Appendix 1). Following incubation overnight at 4°C, tissues were washed for 30 min in fresh saline to remove non-specific staining (Stuart et al., 1974). Tissue whole mounts were examined and photographed under a Wild binocular and a Leitz Dialux compound microscope. Agfa Ortho 25 black and white film (with enhanced sensitivity to red light) and Kodak Ectachrome 64 colour film were used. Drawings were made with the aid of a camera lucida.

4.2.2. Formaldehyde induced fluorescence method

4.2.3 Preparation of tissues

The method described by Falck and Owman (1965) was used. The CNS, frontal ganglia, corpora cardiaca and corpora allata were quickly dissected out from adult and sixth instar larvae of S. littoralis. These tissues were stretched on coverslips and frozen on an aluminium block, pre-cooled in liquid nitrogen. Head capsules were partially dissected to expose the cerebral ganglia before freezing in small compartments in the metal block. Fast cooling is essential, to avoid ice crystals forming and damaging the tissue.

4.2.4 Pre-treatment with drugs and formamidines

Marsden and Kerkut (1969) recommended injecting L-DOPA (a precursor of biogenic amines) at the rate of 5 µg/g wet weight of insect. A lower concentration of 0.5 µg/g was used in S. littoralis to

avoid non-specific uptake of non-aminergic cells.

Frontali et al. (1968) depleted monoamines in cockroach nervous tissue by two injections of reserpine (50 µg/g) over two days. A similar procedure was adopted in S. littoralis; 5 mg of the drug was disolved in 40 µl acetic acid and made up to 1 cm^3 with saline. Adults were injected twice with 10 µg of reserpine at intervals of 24 h.

Tissues were also incubated in 10^{-6} M tryptamine hydrochloride in saline for one hour as Evans (1978a) reported this compound was a competitor for the sodium sensitive, high affinity uptake mechanism for octopamine in the cockroach nerve cord.

To investigate the effects of formamidines on biogenic amines an LD_{50}^{48} of CDM.HCl was injected into sixth instar larvae. In vitro effects were examined by incubating nervous tissue in a 10^{-6} M solution of this compound.

4.2.5 Freeze drying procedure

The aluminium block with frozen tissue was transferred from liquid nitrogen directly into an Edwards 30P2 centrifugal freeze-drier containing phosphorus pentoxide. The chamber evacuated immediately. After freeze drying for 4-5 days, the temperature was raised in the chamber to 35°C to avoid condensation of water on the lyophilized tissue. Air was allowed into the system via flasks containing dessicant. Specimens were transferred to opaque boxes containing phosphorus pentoxide.

4.2.6 Histochemical procedure

The water content of paraformaldehyde is critically important in the Falck-Hillarp technique (Falck & Owman, 1965). After drying for 10 days over phosphorus pentoxide, a range of humidities was achieved by storing paraformaldehyde over various concentrations of sulphuric

acid. The optimum R.H. varies for different species and the dryness of the specimen. It can only be determined by trial and error. 30, 40, 50, 60, 70, 80 and 90% R.H. were evaluated at 60, 65, 70, 75 and 80 °C. The optimum exposure for S. littoralis was found to be 90% R.H. at 65°C for 1.5 h and these conditions were used routinely. The high humidity gave a strong fluorescent reaction but shows some diffusion. In other insects optimum results were achieved with 70% R.H. (Klemm, 1976). Tissue was exposed to the vapour generated by 5 g of paraformaldehyde in 1 dm^3 containers. Fixation by the gas minimises diffusion of biogenic amines in the tissues.

A control specimen was included for each tissue. This was processed in the same way except that paraformaldehyde was omitted from the reaction vessel.

4.2.7 Embedding and sectioning

Specimens were embedded in degassed, dried paraplast wax (melting point, 56°C) in vacuo over phosphorus pentoxide. This ensured complete and rapid penetration of wax into the tissue in about 1 h.

The presence of chitin in the head capsule compressed sections of less than 15 µm thickness and routinely 15-20 µm sections were cut on a Reichert microtome. Water cannot be used to flatten the ribbon; it would immediately quench FIF. A number of alternative agents were therefore evaluated. Glycerin albumen was originally applied to the slides to increase adherence but this also increased autofluorescence. Sections were thus flattened by briefly reheating on a hot plate. Sections were mounted in dry paraffin, under 0.17 mm coverslips.

Tissues were also sectioned on a Cambridge Rocking microtome in a Bright cryostat at -30°C. The sections were then lyophilized and processed by the Falck-Hillarp and glyoxylic acid methods.

4.2.8 Fluorescence microscopy

Specimens were examined immediately under incident light from a 100 W high pressure mercury lamp in a Leitz Ploempak 2.4 vertical illuminator. This unit was mounted on a Dialux 20 microscope.

Optimum filters for FIF were used; these consisted of an excitation filter (band pass 355-425 nm), used in conjunction with a beam splitting mirror (maximum transmission 455 nm) and a short pass suppression filter, transmitting light above 460 nm. Specimens were also examined with transmitted light from a 200 W lamp in a Zeiss microscope using a BG 38/2-5 and 12/4 exciting and 50 nm barrier filter.

Epi-illumination was found to be superior because there was no light loss through scattering or absorption by the specimen. There were, however, two disadvantages; an increase in autofluorescence especially from chitin and specific fluorescence faded more rapidly.

4.2.9 Differentiation of specific fluorescence from autofluorescence

(1) Control sections were compared with those treated with formaldehyde vapour.

(2) The sodium borohydride specificity test was used (Pearse, 1972) which reduces 4-dihydro compounds to non-fluorescent tetra hydro derivatives. Sections were washed with 100% propan-1-ol to remove liquid paraffin, treated with 0.5% (wt/vol) sodium borohydride in 95% propan-2-ol for 1-10 min followed by a final wash in 100% propan-1-ol (1 min). This treatment quenches amine-specific fluorescence but this can be regenerated by further exposure to formaldehyde. Autofluorescence remains unchanged.

(3) Treatment with reserpine should deplete amine-specific fluorescence but autofluorescence remains unchanged.

(4) Specific fluorescence fades after 24 h or after exposure to ultra-violet light.

4.2.10 Glyoxylic acid method for demonstration of biogenic amines

The technique described by Lindvall and Bjorklund (1974) was followed. Thirty µm cryostat sections and stretch mount preparations were immersed in various concentrations of glyoxylic acid (0.1-10% w/v) at 4°C in 0.1 M phosphate buffer, pH 7.0. Specimens were left in the solution for up to 5 min. Excess glyoxylic acid was removed, the slides dried rapidly at 60°C by a steam of hot air for 15 min, followed by heating at 100°C for 5 min. The preparations were mounted in dry liquid paraffin and examined immediately.

4.2.11 Photography

Sections were photographed using a Wild MPS 50 camera using black and white Film (Ilford Pan F). Films were developed using Acutol to give a fine grain negative. Agfachrome colour film, the optimum for catecholamine fluorescence, was processed by Agfa. Exposure times was determined by trial and error.

4.3 Results

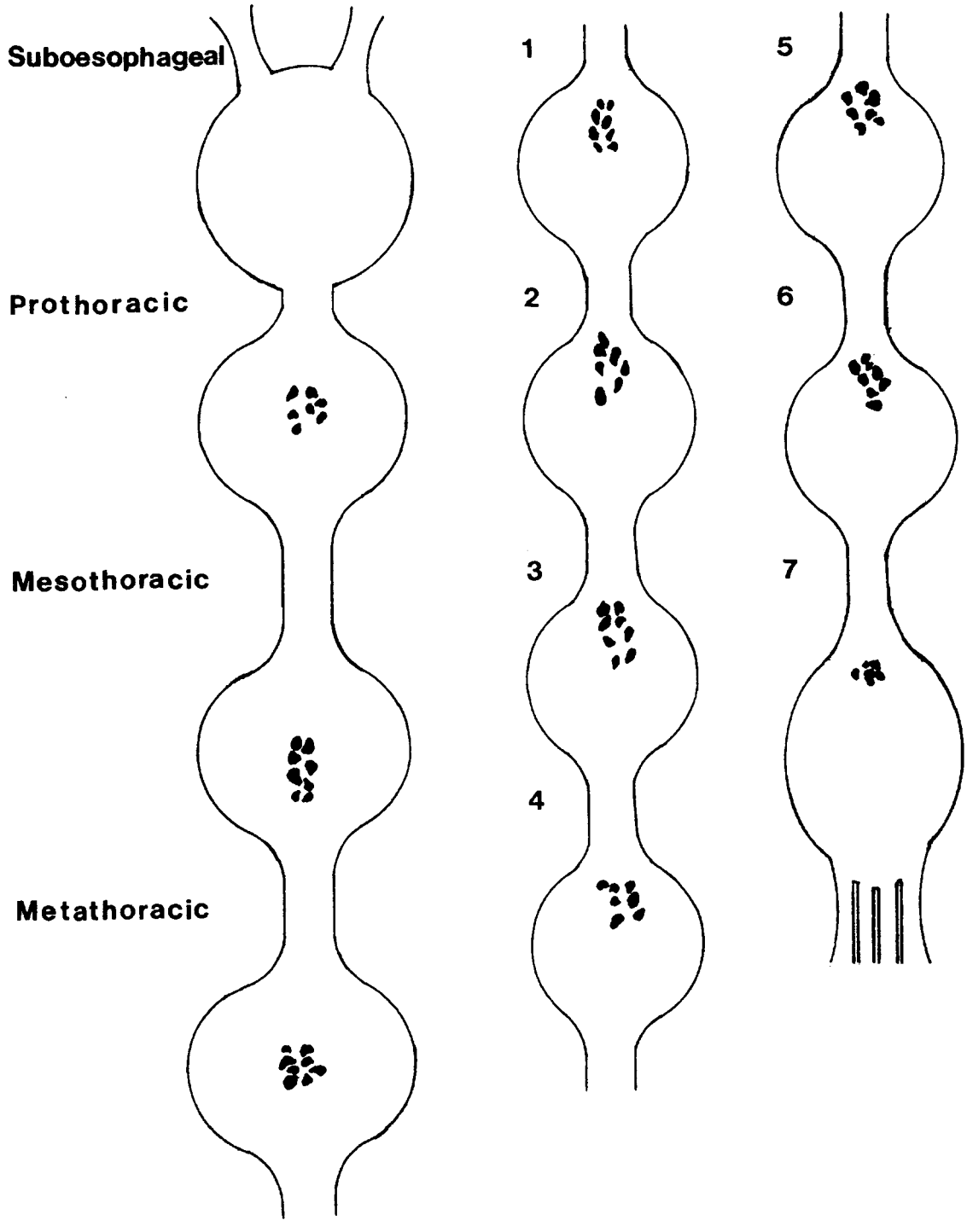
4.3.1 Neutral red staining

The ventral nerve cord of S. littoralis larvae is flattened dorso-ventrally and consists of the sub-oesophageal ganglion, 3 thoracic (pro, meso and metathoracic) ganglia and 7 abdominal ganglia.

Neutral red staining revealed a median group of cells on the dorsal surface of each thoracic and abdominal ganglia (Fig. 6).

The number of intensely staining cells was variable and masked by pigmentation of the tissue and unspecific staining of the sheath. In the pro, meso and metathoracic ganglia, up to 8 large cells 30-40 µm in diameter stained either in the centre or towards the anterior edge (Plates 6 and 7). Groups of smaller cells were sometimes stained. In the 7 abdominal ganglia, 7-8 large cells stained towards the anterior,

Fig. 6 Stylised diagram of the ventral nerve cord of a sixth instar larvae illustrating the groups of dorsal median cells revealed by Neutral red staining. All transverse nerves have been omitted.



occasionally 2 towards the posterior edge and between 2 and 4 on the ventral surface (Plate 8).

Cells staining with Neutral red were difficult to identify in the adult ventral nerve cord because of changes in morphology after metamorphosis. The sub-oesophageal ganglion is reduced in size, the meso and metathoracic ganglia are joined together and the last 3 abdominal ganglia are fused. In adult Lepidoptera the ventral nerve cord is associated with a dorsal vessel which produces a sinuous movement in the CNS, increasing haemolymph circulation (Snodgrass, 1935). This structure also stained with Neutral red, which masked cells staining in the ganglia. A number of cells were present on the dorsal surface of thoracic and abdominal ganglia but the precise location and number could not be determined because of the difficulties already mentioned. Cells staining with Neutral red were also found in the corpora cardiaca (Plate 9), corpora allata and frontal ganglion (Plate 10) of the larvae. These structures were too small in the adult for vital staining of intact tissue.

4.3.2 Distribution of FIF in S. littoralis tissue

The adult brain of S. littoralis, like other insects, consists of an outer cortex of cell bodies which send processes into the neuropile, forming a fibrous mass (Klemm, 1976). Three major areas can be distinguished: the protocerebrum, deutocerebrum and tritocerebrum. The highly structured neuropile of the protocerebrum can be further subdivided into areas such as the corpora pedunculata (mushroom bodies), central body complex and optic lobes.

Green FIF fibres (possibly containing monoamines) were observed throughout the unstructured neuropile of the moth brain. Cell bodies within the protocerebral cortex were strongly fluorescent. FIF was present in areas of the neuropile corresponding to the central body,

and lobes of the mushroom bodies but was absent from all other regions of the protocerebrum neuropile such as the calyx and protocerebral bridges (Plate 12).

The neuropile of the deutocerebrum comprises the antennal lobe and an area receiving mechanosensory information. FIF was also detected in this region.

The tritocerebrum connects the brain with the suboesophageal and frontal ganglion. Sensory fibres from the anterior mouth parts enter the tritocerebrum with the frontal nerve. The tritocerebrum of S. littoralis contained a number of single fluorescent cell bodies and many cell fibres (Plate 13).

Insect optic lobes consist of three regions of integrative neuropile, the lamina, medulla and lobula which connect the compound eye to the cerebral ganglion. Compound eyes are absent from holometabolous larvae such as S. littoralis. Intense fluorescence was arranged into bands in the medulla (Plates 14 & 15). Weak FIF was also present in the lobula and lamina. FIF was also present in the antennal lobe (Plate 16).

No fluorescent cell bodies were detected in adult or larval sub-oesophageal, thoracic or abdominal ganglia. FIF was restricted to the neuropile (Plates 19 & 20). Fluorescent cell bodies could not be detected in larval frontal ganglion, corpora cardiaca or corpora allata. FIF was, however, present in the neuropile of the frontal ganglion.

4.3.3. Pre-treatment with drugs and formamidines

In some of the preparations from adult cerebral ganglion, reserpine reduced or abolished FIF in the neuropile, but the intensity of cell body fluorescence remained unchanged. No quantitative change in fluorescence intensity could be detected after injection of L-DOPA or

chlordimeform. Some fluorescence may be associated with the dorsal median cell bodies following incubation with tryptamine (Plate 21).

4.3.4 Differentiation of specific fluorescence from autofluorescence

Insect tissue, particularly chitin fragments and tracheoles, displayed autofluorescence when processed without formaldehyde treatment (Plate 17). This could readily be distinguished from FIF by intensity and colour (Plate 16). Sodium borohydride quenched FIF which could be regenerated by further exposure to formaldehyde. This was achieved with some difficulty owing to detachment of specimens from the slide and deterioration of the section. Autofluorescence remained unchanged in tissue treated with reserpine. FIF faded rapidly on exposure to ultra-violet light.

4.3.5 Glyoxylic acid method for demonstration of biogenic amines

No specific fluorescence could be detected in either stretch mount preparations or cryostat sections of larval and adult ventral nerve cords or cryostat sections from cerebral ganglia. Condensation of biogenic amines with glyoxylic acid proceeds in a similar manner to formaldehyde but the fluorescence is much brighter (Lindvall & Bjorklund, 1974). In addition to improved sensitivity, a major advantage is that tissue can be processed within fifteen minutes without lyophilization and distortion of specimens.

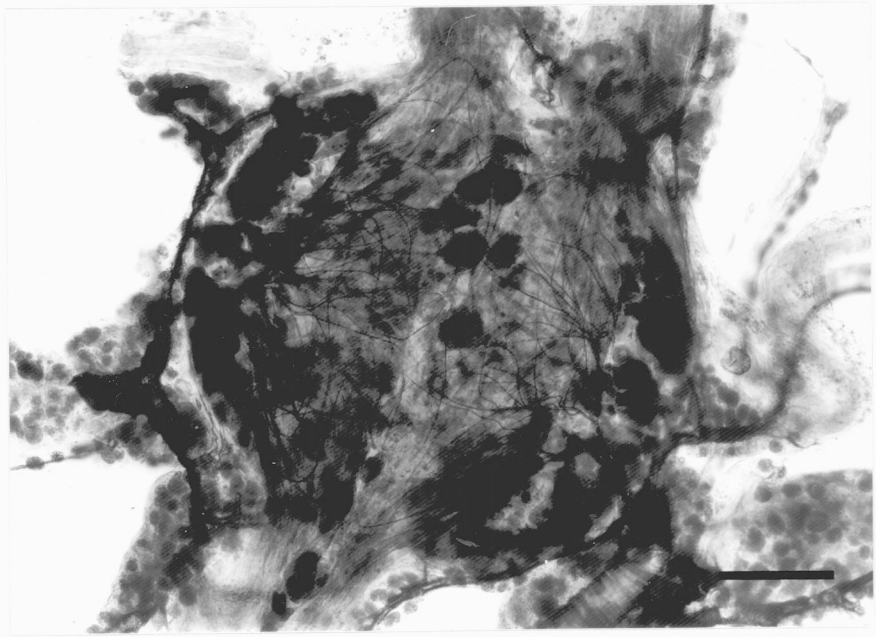
Although fluorescence was found in mouse iris and mesentery, it could not be detected in S. littoralis tissue. Autofluorescence was much brighter and could have obscured specific fluorescence. Alternatively, the glyoxylic acid might be prevented from penetrating the tissue by the embedding medium. Klemm (1980) in a recent review of the histochemical demonstration of biogenic amines reports the

PLATE 6. Light micrograph of the dorsal surface of a sixth instar mesothoracic ganglion showing the cells revealed by Neutral red staining. The anterior edge of the ganglion is towards the top of the picture.

Scale bar = 100 μm.

PLATE 7. Light micrograph of the dorsal surface of a sixth instar metathoracic ganglion; orientation as in Plate 6.

Scale bar = 100 μm



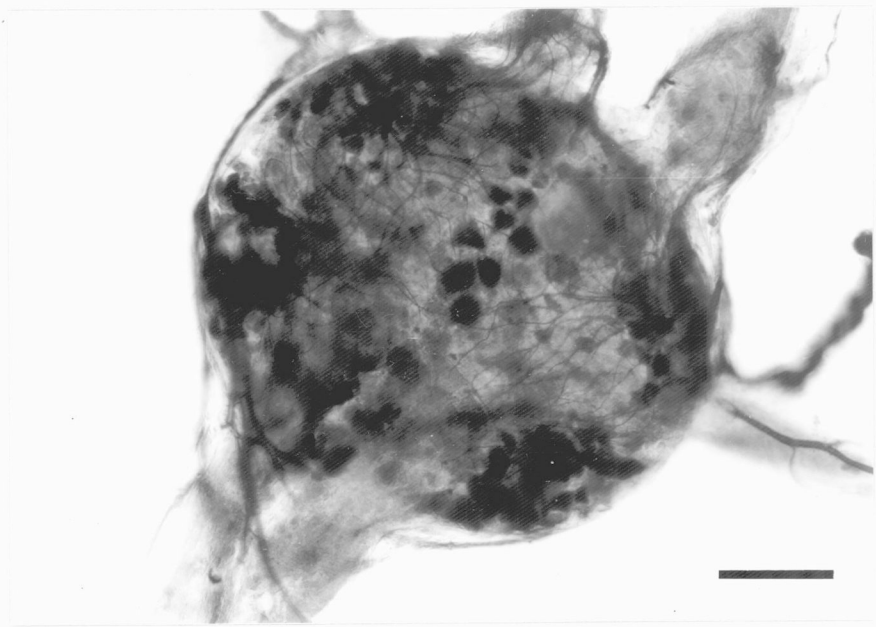
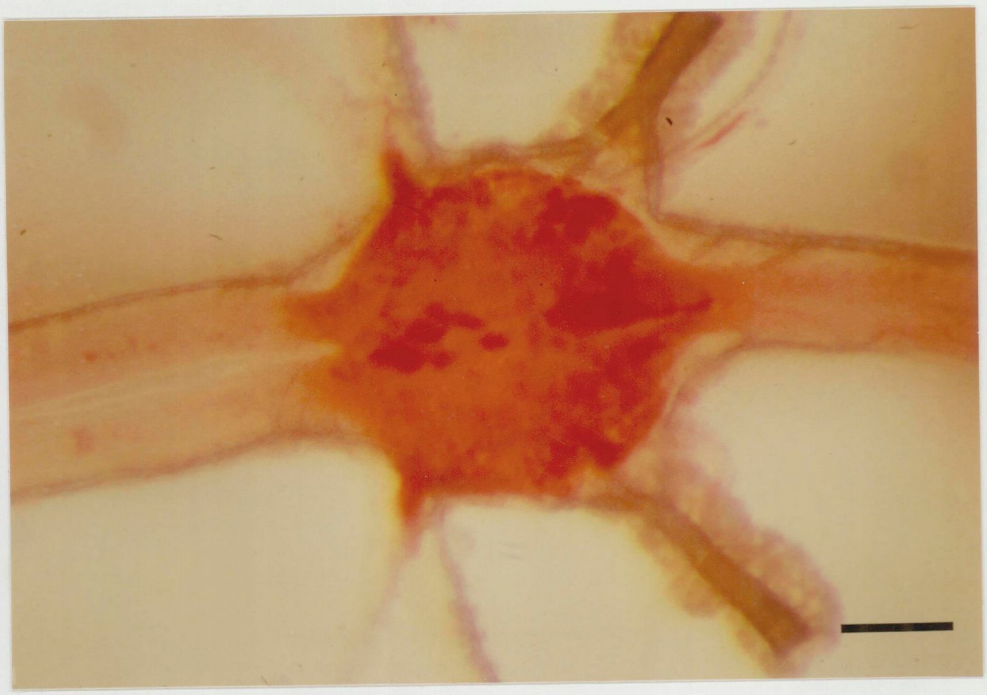


PLATE 8. Light micrograph of the dorsal surface of a sixth instar 2nd abdominal ganglion showing the cells revealed by Neutral red staining. The anterior edge of the ganglion is to the left of the photomicrograph.

Scale bar = 100 μm.

PLATE 9. Light micrograph of the corpora cardiaca from a sixth instar larvae stained with Neutral red. Nerves connecting the corpora cardiaca to the cerebral ganglia can be seen at the top of the picture.

Scale bar = 50 μm.



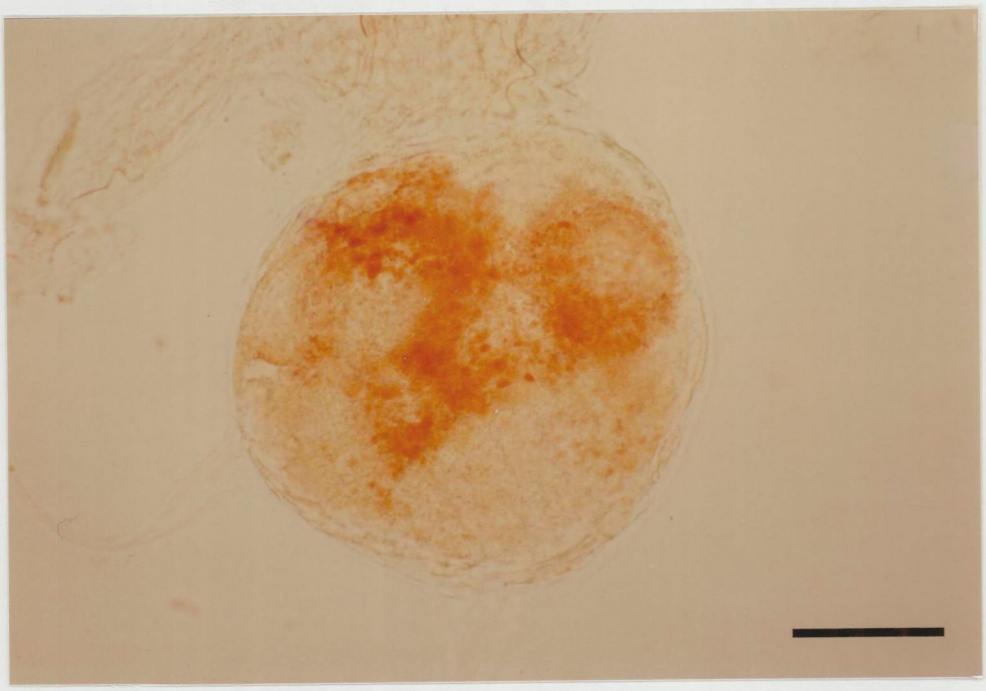
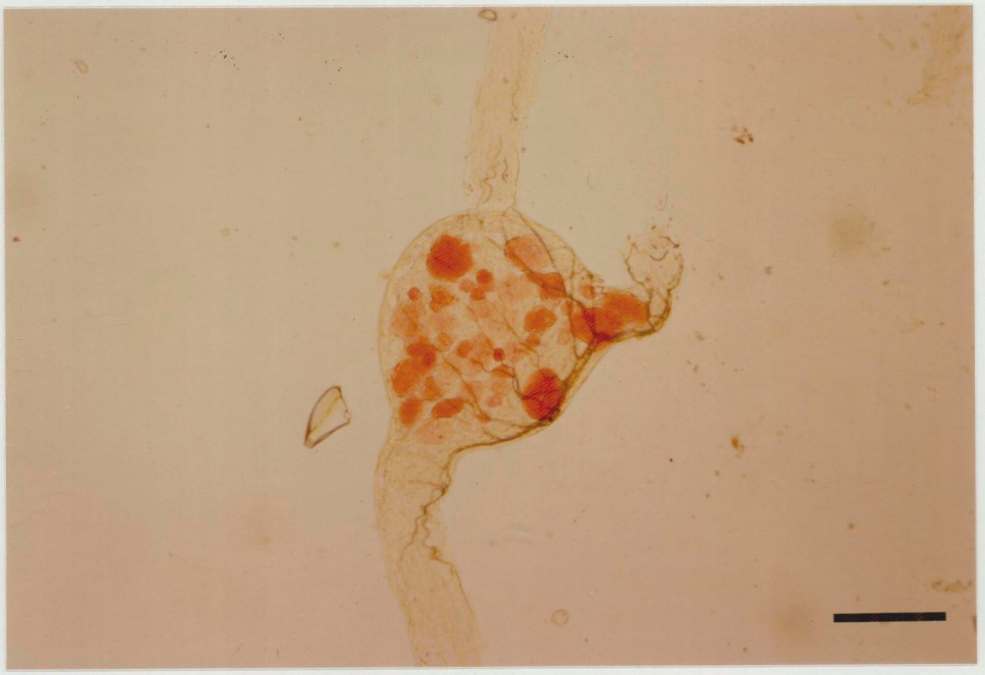


PLATE 10. Light micrograph of the frontal ganglion from a sixth instar larvae stained with Neutral red. The two frontal commissures connecting the ganglion to the tritocerebrum exit towards the top and bottom of the photomicrograph.
Scale bar = 100 µm.

PLATE 11. Light micrograph of sixth instar cerebral ganglion after Neutral red staining. Posterior view with the dorsal edge towards the top of the picture.
Scale bar = 100 µm.



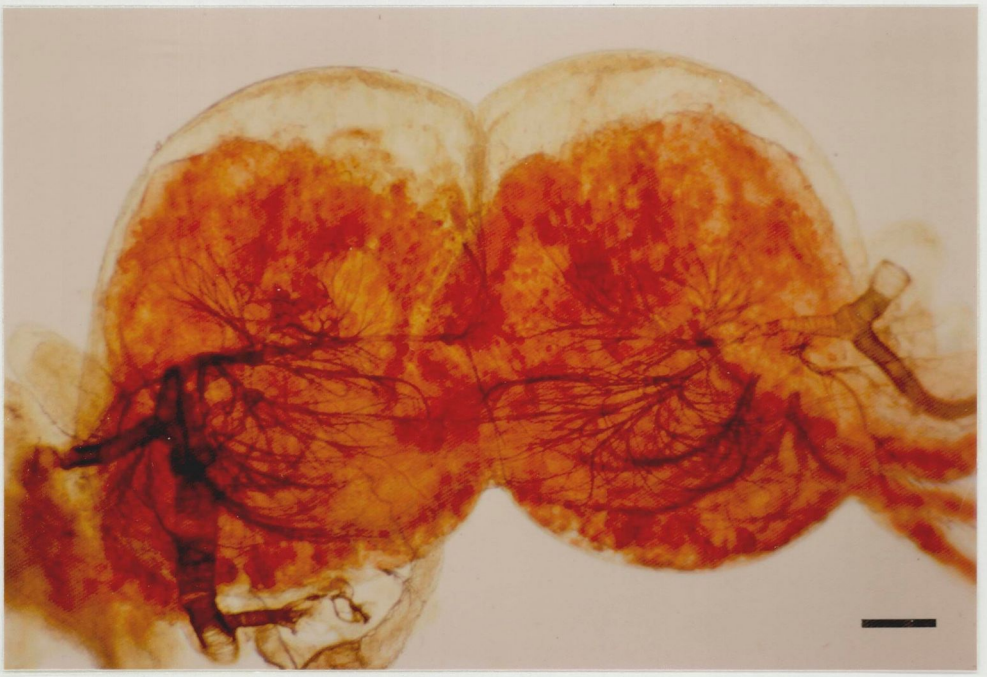
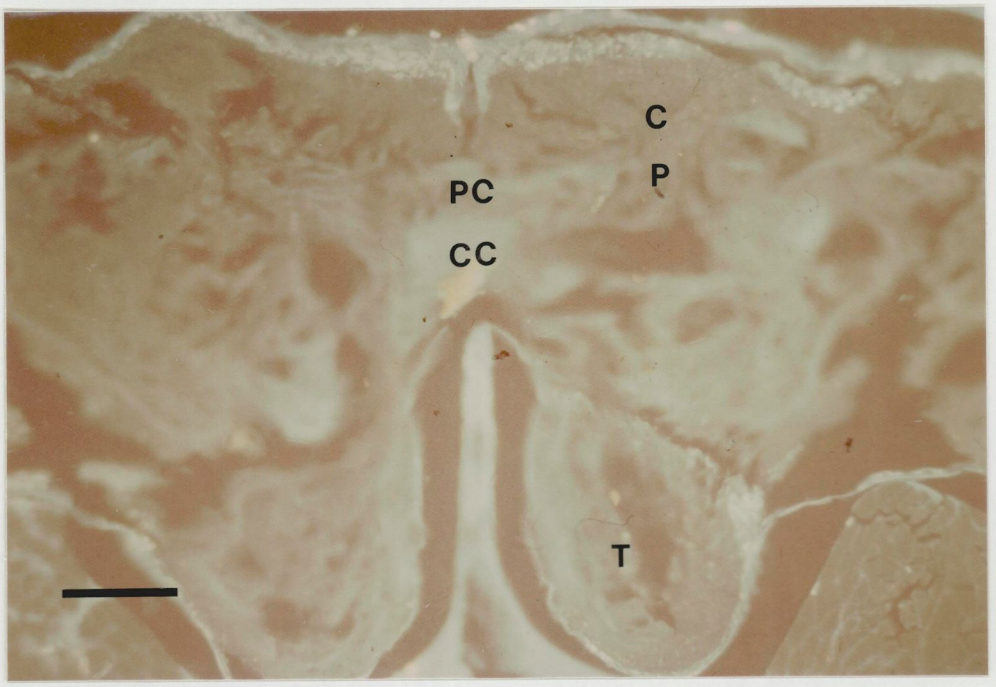


PLATE 12. Light micrograph of FIF in a transverse section of an adult S. littoralis cerebral ganglion. Note intense fluorescence of unstructured neuropile. The dorsal surface is towards the top of the photomicrograph, the optic lobes (not illustrated) to the left and right. C. - calyx; C.C. - central body complex; P. - pedunculus; P.C. - pons cerebralis; T - tritocerebrum.
Scale bar = 100 μm.

PLATE 13. Light micrograph of a transverse section of an adult male tritocerebrum. Note FIF containing cell bodies in the cortex and fibres in the neuropile.
Scale bar = 50 μm.


C
P
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T

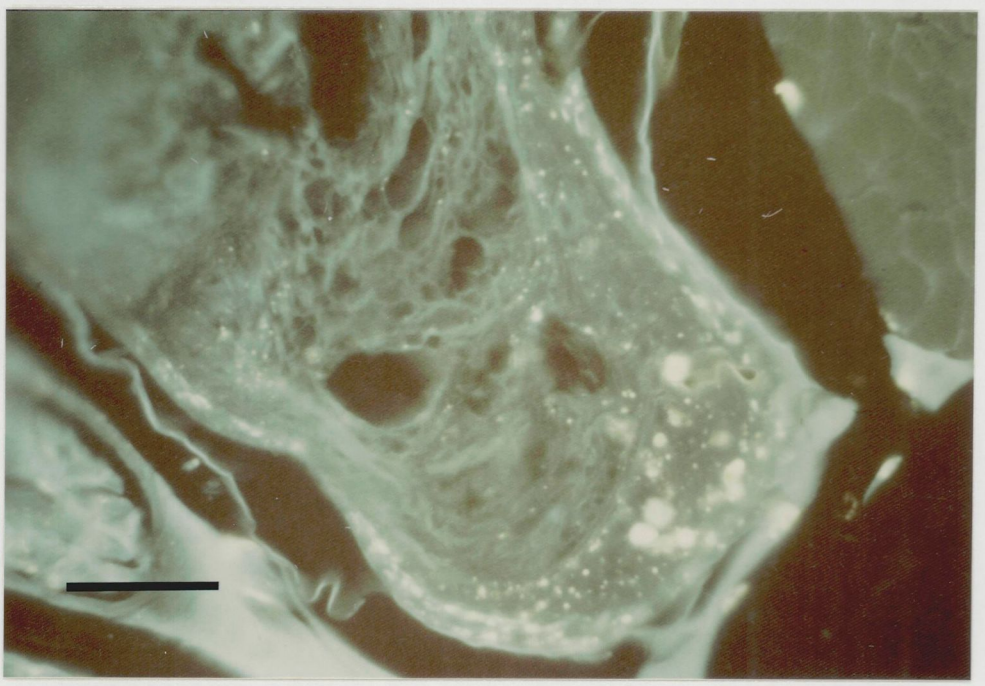
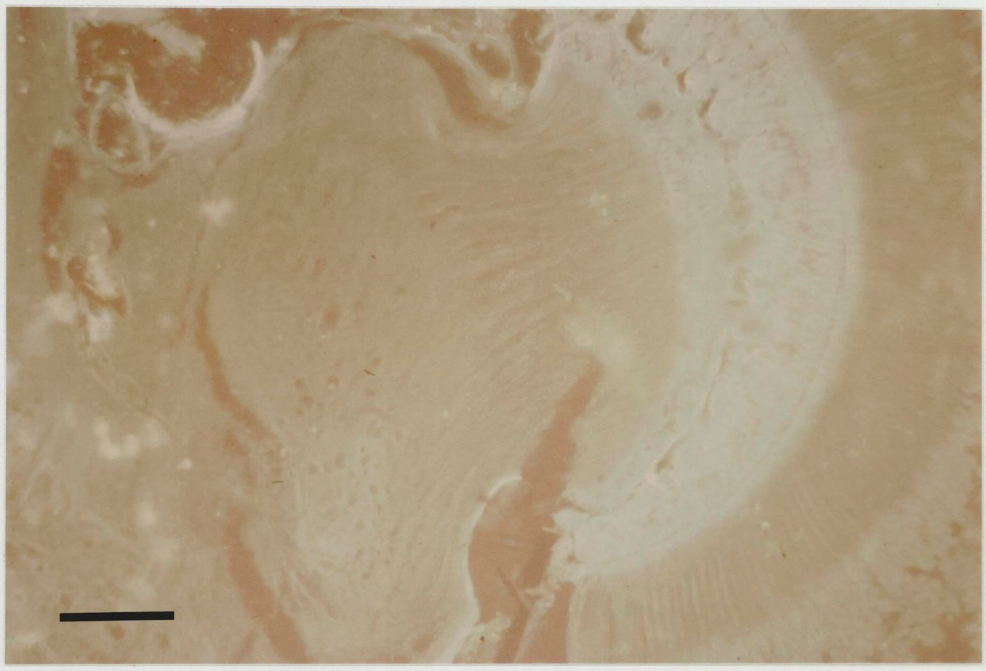


PLATE 14. Light micrograph of FIF in transverse section through the adult male optic lobes.
Note intense fluorescence of medulla.
Scale bar = 100 μm.

PLATE 15. FIF in cell bodies and fibres in the adult optic lobe.
Scale bar = 50 μm.



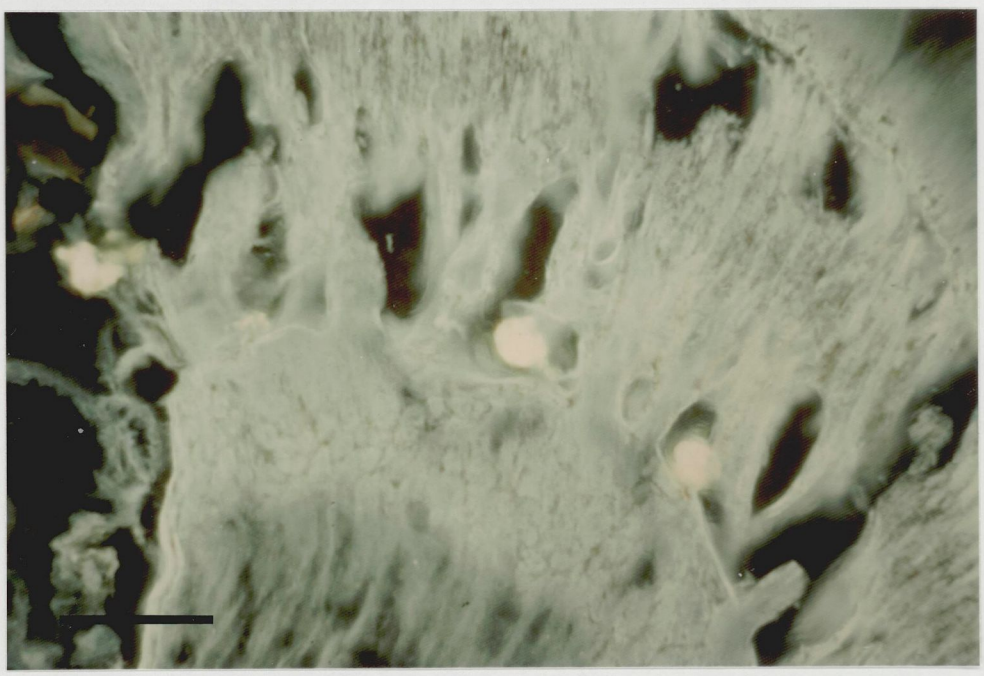


PLATE 16. FIF fibres within the adult antennal lobe.

Scale bar = 100 μm.

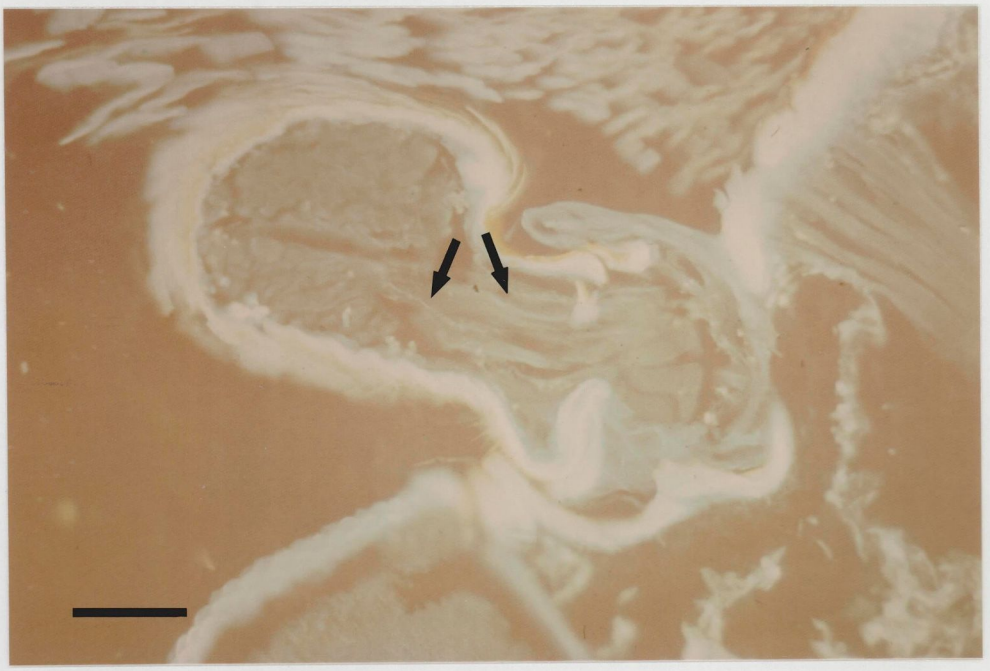
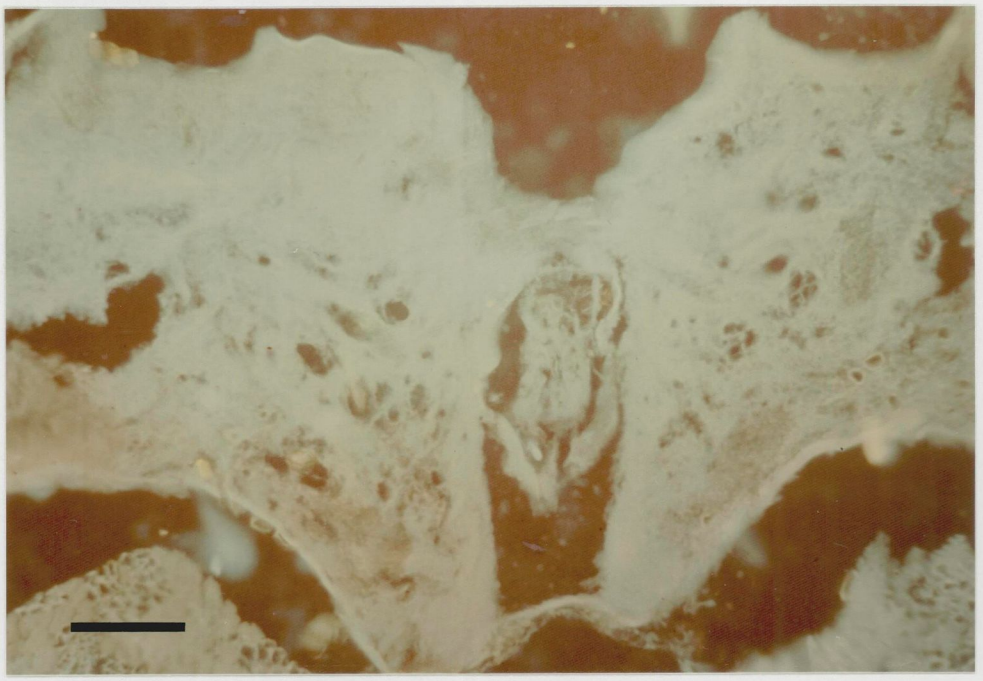


PLATE 17. Transverse section of an adult cerebral ganglion quenched with sodium borohydride after FIF treatment, illustrating autofluorescence. The dorsal surface is towards the top of the picture. Compare with Plate 12.

Scale bar = 100 μm.

PLATE 18. Transverse section of the optic lobe processed without exposure to formaldehyde. Compare with Plate 14.

Scale bar = 100 μm.



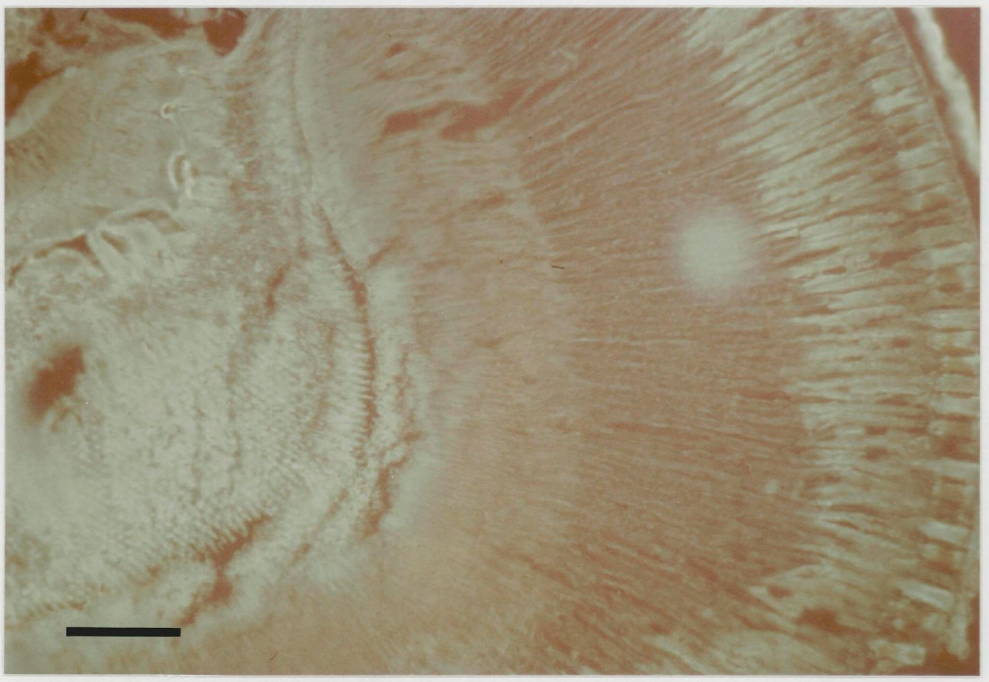
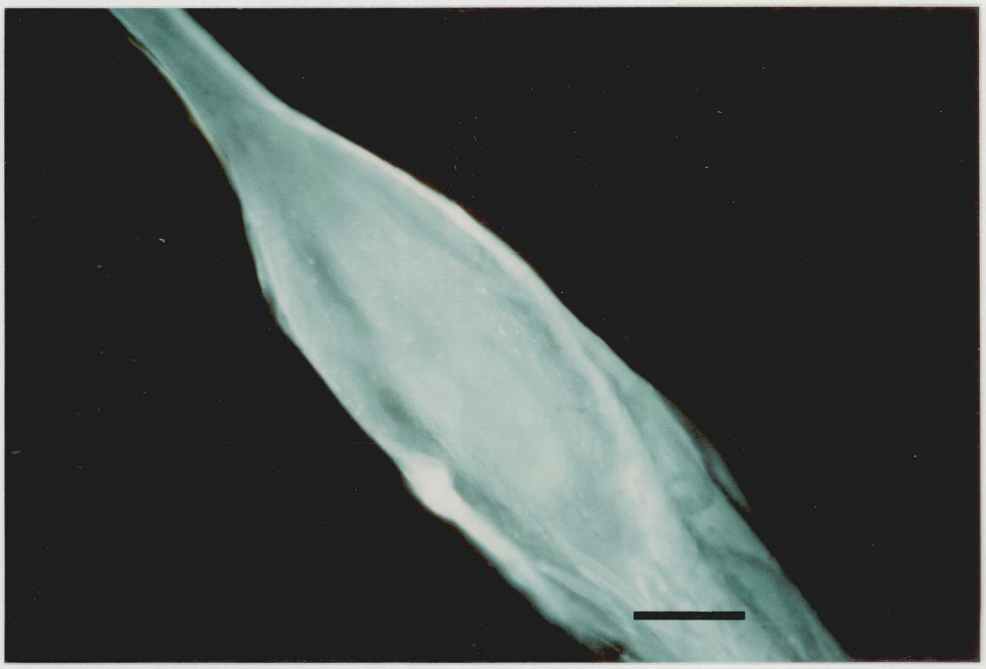


PLATE 19. Light micrograph of the dorsal surface of an adult terminal ganglion. The FIF is confined to the neuropile. The anterior edge is towards the top of the photomicrograph (and in Plate 19 and 20).

Scale bar = 100 μm.

PLATE 20. Light micrograph of the dorsal surface of a 2nd abdominal ganglion from a sixth instar larvae. FIF is confined to the neuropile and cannot be detected in dorsal median cell bodies revealed by Neutral red (Plate 6).

Scale bar = 100 μm.



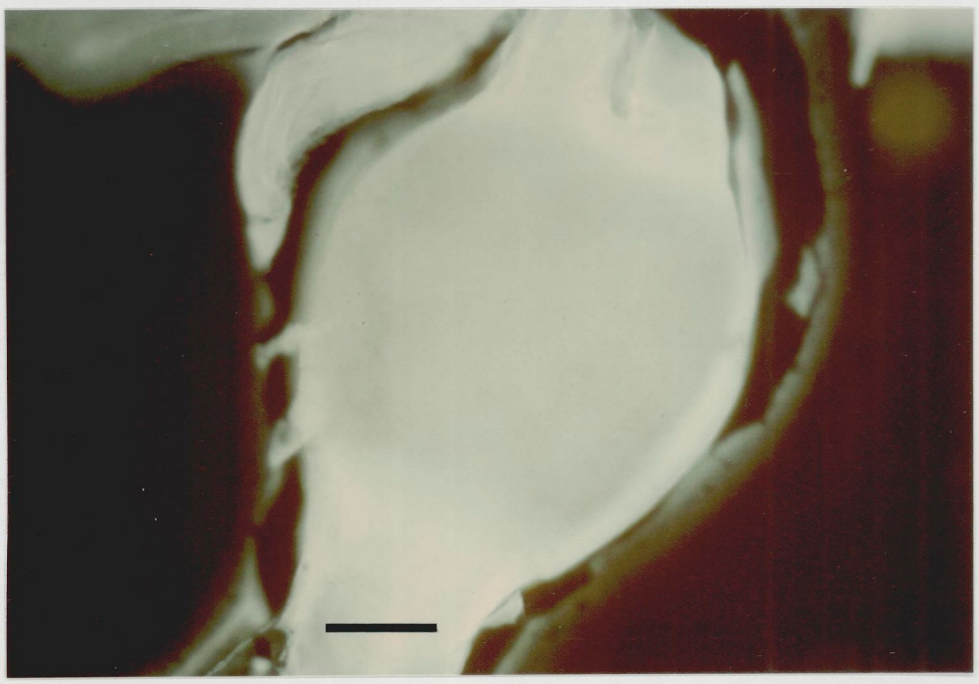
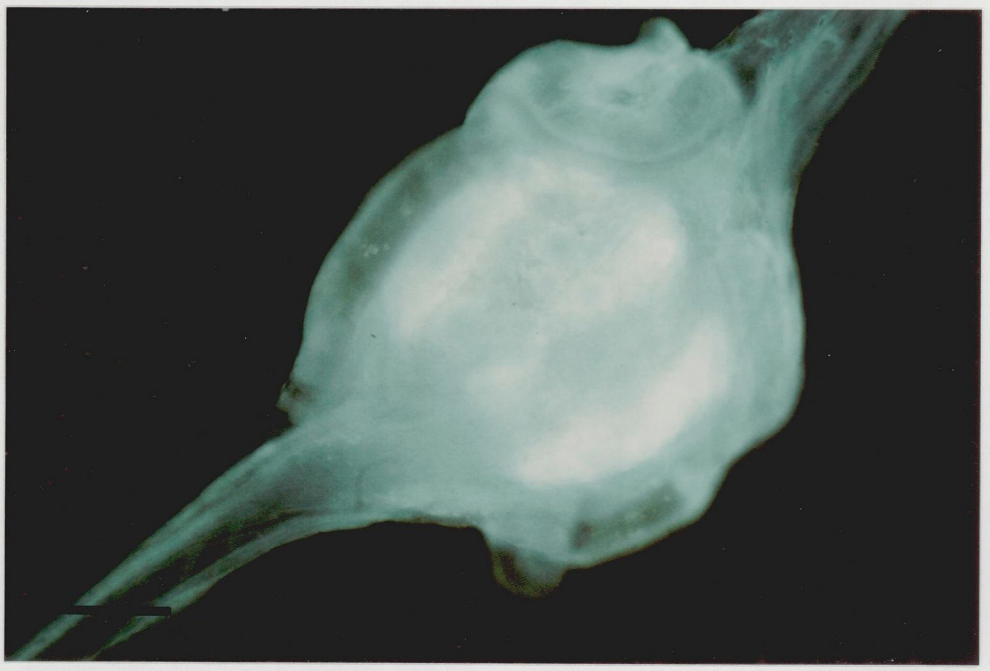


PLATE 21. Light micrograph of FIF in a 2nd abdominal ganglion following incubation with tryptamine. Note slight fluorescence in dorsal median cell bodies.

Scale bar = 100 µm.



glyoxylic acid technique has not yet been applied successfully to insects.

4.4 Discussion

4.4.1 Biogenic amines in S. littoralis tissue: Neutral red

The group of cells revealed by Neutral red staining in the thoracic and abdominal ganglia of S. littoralis may correspond to cells of similar morphology in other insects (Evans, 1980a).

In the locust, a group of dorsal unpaired median (DUM) neurones have been described which have bifurcating axons projecting symmetrically into the left and right peripheral roots of the ganglia. This feature distinguishes them from the majority of locust motoneurones which are bilaterally paired and have axons leaving the central ganglia in the nerve roots of only one side (Evans, 1980a). The cells are unusual having soma which generate large over-shooting action potentials of 60-110 mV, typical of neurosecretory neurone action potentials (Evans, 1980a). DUM cells stain selectively with Neutral red and one of this group in the locust metathoracic ganglion is the DUMETi neurone containing octopamine (Section 1.5).

Unlike cockroaches and locusts, some if not all the dorsal median cells in S. littoralis appear to be paired. The anterior group may be identical to the 4 pairs of large cells (at least 20μm in diameter) on the dorsal surface of fourth instar M. sexta abdominal ganglia (Taylor & Truman, 1974). They were also present in the pharate adult but were reduced to 3 pairs. Two pairs of ventral cells located towards the posterior edge have an identical branching pattern to locust DUM neurones, sending axons out of both nerve roots of the ganglia. A second pair of median cells on the dorsal surface bifurcate to give a branch in the left and right connectives, exiting to the muscle of the body wall via the dorsal segmental nerve of the next abdominal ganglia

(Taylor & Truman, 1974). Casaday and Camhi (1976) also describe median cells in the thoracic ganglia of this moth.

Evidence is accumulating for the presence of dorsal median neurones in Lepidoptera. Although they have not been characterised physiologically or assayed for octopamine, combining the results from the radioenzyme assay and histochemistry suggest the amine may be located in the median cells of S. littoralis. It is possible there is an equivalent cell to Dumeti which modulates Lepidopteran muscle. Modulation of certain flight muscles by octopamine in adult M. sexta has already been mentioned (section 1.5). The incessant flight of adults and hyperactivity of larvae may result from formamidines stimulating octopamine receptors and mimicking the neuromodulatory role of octopamine.

Stimulation of unidentified neurones within the CNS of M. sexta would also contribute to the observed hyperactivity. The function of octopamine within the brain and ventral nerve cord is still uncertain and whether formamidines also mimic the actions of this amine in the CNS has not been investigated.

The insect somatogastric nervous system is responsible for a number of functions, such as control of peristaltic movement of the foregut, control of salivary release and protein synthesis. In S. littoralis, the somatogastric nervous system consists of a single frontal ganglion, connected by two frontal commissures to the tritocerebral lobes. An unpaired recurrent nerve containing motoneurones connects the frontal ganglion to the crop and foregut. The cells staining with Neutral red did not fluoresce suggesting they contained a non-fluorescent amine. Although the octopamine content was not measured, the locust somatogastric nervous system contains a relatively high concentration of octopamine (David & Lafon-Cazal, 1979). The physiological significance of this is uncertain. The effects of

various biogenic amines on myogenic contractions of gut muscle have been investigated. Although octopamine has not yet been tested Evans (1980a) believes octopamine would inhibit myogenic activity. If this were the case, it would provide a further target for formamidines to effect feeding behaviour.

4.4.2 Biogenic amines in S. littoralis tissue: FIF

The optimum conditions for the development of FIF in S. littoralis tissue were unusual, namely 90% R.H. paraformaldehyde heated to 65°C. Although the strongest fluorescent condensation reaction is obtained at 80-95% R.H. (Falck & Owaran, 1965) the fluorescent structures are often diffuse. 70% R.H. is usually sufficient for insect tissue (Marsden & Kerkut, 1969). A temperature of 80°C, for 1-2 h has often been used to develop FIF but in S. littoralis tissue this resulted in increased autofluorescence. Although attempts have been made to use the FIF method for semi-quantitative analysis, the fluorescence intensity varied between tissue processed in the same batch. Only drastic pharmacological treatment (for example reserpine) consistently altered fluorescence. CDM did not appear to act like reserpine (to deplete monoamines) or like L-DOPA (to enhance fluorescence) but this was a subjective judgement.

A number of monoamines can be distinguished using the fluorescent microscope. Catecholamine fluorophores (for example, dopamine, noradrenaline) are green or yellow green. Indolealkylamines (for example, 5-HT) are yellow. The FIF observed in S. littoralis tissue is unlikely to result from reaction with a secondary amine such as adrenaline. These fluorophores require at last 3 h of exposure to formaldehyde at a relatively high temperature to develop and would be extracted by organic solvents when mounted (Falck & Owman, 1965).

The distribution of FIF in adult S. littoralis cerebral ganglia and optic lobes was similar to results reported for this insect by Klemm (1976). However, Klemm (1979) found two pairs of three weakly fluorescent cell bodies in the adult frontal ganglion.

The absence of FIF from the region of the protocerebral neuropile corresponding to calyx and pedunculus may be significant. This area does not fluoresce in the cockroach (Klemm, 1976), but does stain with Neutral red. The median group of cells on the dorsal surface of the abdominal and thoracic ganglia also stained with Neutral red suggesting they were aminergic and failed to fluoresce when treated with formaldehyde.

In both these cases, the cells may not have contained sufficient catecholamines to fluoresce. This seems unlikely, since the technique can detect as little as 5×10^{-6} pM of amine (section 4.1). Alternatively the cells could contain a non-fluorescent amine such as octopamine. Dorsal median neurones of the locust (Evans & O'Shea, 1978) and cockroach (Dymond & Evans, 1979) also stained selectively with Neutral red but did not fluoresce when treated with formaldehyde. The presence of octopamine was confirmed by radioenzymic assay on single physiologically identified cells in the locust (Evans & O'Shea, 1977, 1978) and groups of unidentified soma from the cockroach (Dymond & Evans, 1979).

Two pairs of dopamine-containing cell bodies have been discovered in Tricoptera thoracic and abdominal ganglia (Klemm, 1971). Their position was variable and it was not clear whether they were homologous to Neutral red staining cells in Orthoptera. With this exception, localizing cell bodies containing FIF in the ventral nerve cord have not been successful in other insects (Evans, 1980a).

The radioenzymic assay (section 5.3) confirmed the presence of octopamine in the ventral nerve cord of adult and larval S. littoralis

although individual cells were not dissected out and assayed. The results provide circumstantial evidence that some of the dorsal median cells contain a non-fluorogenic amine which might be octopamine. These may be analagous to the DUM cells which have been characterised in locusts and cockroaches.

FIF has previously been detected in the corpora cardiaca of locusts and cockroaches (Klemm & Falck, 1978). The presence of dopamine and a small amount of noradrenaline has been confirmed by radioenzymic assay in the cockroach (Dymond & Evans, 1979). Octopamine has also been found in much higher concentrations in the corpora cardiaca of both insects (Evans, 1978b).

Although a number of cells in S. littoralis corpora cardiaca stained with Neutral red, FIF could not be detected. These results support the findings of Klemm and Falck (1978) who were unable to demonstrate FIF in the neurosecretory system of this moth. They did not consider the possibility that the neurohemal tissue contained a non-fluorogenic amine such as octopamine which in S. littoralis may fulfil the functions of catecholamines in the corpora cardiaca of other insects.

4.4.3 Function of biogenic amines in neurohaemal organs in relation to the effects of formamidines

The association of biogenic amines and neurohaemal tissue has been known for some time but the function remains uncertain (Evans, 1980a). Amines may serve as releasing factors for peptide hormones. Alternatively amines might accumulate in neurohaemal organs as the most efficient site for rapid release into the blood. Evans (1978b) points out in the cockroach, octopamine is highly concentrated in neurohaemal tissue. When compared on a volume basis, there was 700 times more octopamine in the abdominal medial neurohaemal tissue and 200 times more in the corpora cardiaca than in the metathoracic ganglion.

The potential neurohaemal role of octopamine is supported by the presence of this phenolamine in the haemolymph of L. migratoria (David & Lafon-Cazal, 1979), S. gregaria (Goosey & Candy, 1980) and S. littoralis (section 5.3). The source of the circulating amine is unknown.

Some of the sub-lethal effects of formamidines could result from mimicking the neurohormonal actions of octopamine in S. littoralis. Many physiological effects of octopamine are consistent with the compound being released under conditions of stress, altering carbohydrate and lipid metabolism. These include an increase in glycogen phosphorylase activity and stimulating glycolysis in the cockroach nerve cord (Robertson & Steele, 1972). An increase in glucose oxidation in locust flight muscles (Candy, 1978) and an increase in sodium dependent respiration in the nerve cord (Steel & Chan, 1980).

An excitation-induced hypertrehalosemic (EXIT) response has been investigated by Downer (1979a) and octopamine has been suggested as the probable mediator of this response (Downer, 1979b). Octopamine was the most potent amine tested producing hypertrehalosemia after injection and it was capable of increasing trehalose production from isolated fat body and elevating cAMP (Gole & Downer, 1979).

Excitation of locusts by handling after being left undisturbed for 16 h induced a rapid rise in haemolymph octopamine and lipid cells within minutes. This persisted for 1-2 h. Excitation-induced hyperlipemia was reduced by phenoxybenzamine and potentiated by octopamine suggesting the rise of both compounds was causally related (Orchard & Loughton, 1981). Octopamine stimulated isolated fat body to release lipid, with a threshold of 2×10^{-7}M and maximum activity at 5×10^{-6}M. The threshold for a response was close to the observed haemolymph octopamine concentration.

Fat body receptors showed similar pharmacological properties to other octopamine receptors such as antagonism with phentolamine and phenoxybenzamine but were unaffected by the β-blocker, propanalol. AKH and octopamine both act on the fat body to elevate cAMP but only the action of octopamine could be blocked by phentolamine, suggesting the presence of two different receptors (Orchard, 1982).

In S. gregaria, Goosey and Candy (1980) found a rapid rise in haemolymph octopamine, during the first 10 minutes of flight. Haemolymph lipid was also elevated but circulating AKH levels had not risen at this stage (Cheesman & Goldworthy, 1979). At the commencement of flight octopamine liberated into the haemolymph acts on the fat body to release lipid (Goosey & Candy, 1980).

Although further investigations are necessary, the results are consistent with formamidines mimicking this neurohaemal role. Singh et al. (1981) believe CDM and DCDM act on post-synaptic octopamine receptors in the glandular lobe of the corpora cardiaca inducing the release of hyperlipemic hormone. In S. littoralis, octopamine is present in the haemolymph and may be associated with the corpora cardiaca. Changes in lipid metabolism could occur by a mechanism similar to that in the locust. Whether changes in carbohydrate metabolism are brought about by an "EXIT" response mediated by octopamine remains to be investigated.

Chapter 5

RADIOENZYMATIC ASSAY FOR OCTOPAMINE

5.1 Introduction

The octopamine content was estimated by the method of Molinoff et al. (1969) as modified for insect tissue by Evans (1978b). This method is based on the conversion of endogenous octopamine to labelled synephrine using S-adenosyl-L-[methyl-^{3}H] methionine (SAM) as the methyl donor. The conversion is catalysed by phenylethanolamine-N-methyl transferase (PNMT) partially purified from bovine adrenal glands. As little as 10 pg of DL octopamine can be detected using this procedure.

5.2 Materials and Methods

5.2.1 Tissue extraction

Tissue was dissected from sixth instar larvae and adult male S. littoralis and weighed using a Beckman LM500 microbalance.

Two methods for extracting octopamine were compared. In one the tissue was placed in 25 µl of 0.01 N formic acid, crushed with a glass rod before freezing and thawing five times. Alternatively, tissues were homogenised in 0.02 M tris-HCl buffer, heated in a microtube at 95°C for 3 min before centrifuging at 10,000 g for 5 min in a Hawksley microcentrifuge. No difference was observed between the two methods and extraction with formic acid was used routinely. In preliminary experiments, MAO inhibitors were added to the tissue prior to extraction but this was found to have no effect on octopamine levels.

5.2.2 Assay procedure

Samples from each tissue were run in duplicate tubes with an internal standard in one of the tubes to estimate the recovery of

octopamine. The reaction tubes contained the following:

	TISSUE SAMPLE	TISSUE SAMPLE & INTERNAL STANDARD	STANDARDS	BLANK
	µl	µl	µl	µl
PNMT	10	10	10	10
0.1M Tris HCl buffer	50	50	50	50
Internal standard	-	10	10	-
Tissue sample	10	10	-	-
0.01 N Formic Acid	10	-	10	20
SAM	0.5	0.5	0.5	0.5
Total	80.5	80.5	80.5	80.5

Between 120 and 150 tubes were run in each assay. For 150 tubes, 75 µl of SAM (typical activity between 67-71 Ci/mmol (2.8-6.63 Tbq/mmol) was added to 7.5 cm^3 of 0.1 M Tris HCl buffer and 1.5 cm^3 of PNMT. The reaction mixture was kept cold at all times; 60 µl were added to centrifuge tubes pre-cooled in crushed ice. Ten µl of the samples in formic acid were added, followed by 10 µl of the internal standard (500 pg of DL-octopamine) or 10 µl of formic acid, as appropriate.

A standard curve (with a range of 25 to 2000 pg DL-octopamine) was included in each assay, together with blank tubes. The latter contained 60 µl of the reaction mixture, plus 20 µl of formic acid and were scattered throughout the assay.

Samples were incubated for 30 min. at 37°C without shaking after sealing the tubes with a ground glass stopper. The reaction was ended by the addition of 0.5 cm^3, 0.5 M borate buffer, pH 10.

5.2.3 Extraction procedure

5 cm^3 of toluene/3-methyl-1-butanol (iso-amyl alcohol) 3:2 (v/v) was added to each tube, vortexed for 30 s and then centrifuged for 5 min at room temperature. The organic phase was removed by aspiration and added to 1 cm^3 of borate buffer. The mixing and centrifuging step was repeated. The labelled synephrine was then extracted from 4.5 cm^3 of the organic phase into 0.5 cm^3 of 0.1 M HCl. After vortexing for 10 s the toluene/3-methyl-1-butanol layer was removed and discarded. The aqueous phase was dried down at 80°C under a stream of nitrogen, before adding 5 cm^3 of Bray's scintillant.

5.2.4 Thin layer chromatography of labelled synephrine

The authenticity of the products formed for each tissue was checked by thin layer chromatography (TLC) on silica gel plates (Merck) using the following solvent systems:

(1) propan-1-ol:ammonia:water 80:19:10

(2) butan-1-ol saturated with 1 M HCl.

(3) butan-1-ol:acetic acid:water 4:1:1

TLC plates were dried for 15 min at 60°C cut into strips and sprayed with 0.1 mg/cm^3 solution of fluorescamine in dry acetone containing 4% (v/v) triethanolamine. Standards were visualised under UV illumination; less than 5 µg of authentic standards could be detected (Harmer & Horn, 1976).

Chromatogram strips containing the radioactivity were divided into sections, eluted with 0.1 M HCl and counted in 5 cm^3 of Bray's scintillant.

5.2.5 Partial purification of PNMT

Bovine adrenal glands were frozen in liquid nitrogen immediately after slaughter. All extraction procedures were carried out at 2-4 °C.

The cortex was dissected free of the medulla and discarded. PNMT was extracted from about 60 g of medulla by two different methods.

Using the procedure of Goosey and Candy (1980) the medulla was homogenised in 0.17 M KCl, centrifuged at 36,000 g for 30 min. Fat was removed by aspiration and the supernatant fractionated with ammonium sulphate. The fraction precipitating between 30 and 50% NH_3SO_4 was dialysed overnight against 0.02 M Tris buffer, pH 8.0, with frequent changes of buffer. The contents were concentrated from 30 to 2.5 cm^3 using an Amicon UM10 membrane (retentivity greater than 10,000 molecular weight) in an ultrafiltration cell.

The protein was absorbed onto an o-(diethylaminoethyl) (DEAE) cellulose column DE32 (30 x 1.5 cm, i.d.) previously equilibrated with 0.02 M Tris buffer, pH 8.0.

The column was eluted using a linear gradient between 200 cm^3 of 0.02 M Tris buffer, pH 8.0 and 200 cm^3 0.03 M NaCl in 0.02 M Tris pH 8.0. Separation was monitored with an LKB Uvacord II UV analyser. Fractions (4 cm^3) were assayed with 1 ng of octopamine. Those showing greatest activity were frozen in liquid nitrogen and stored at -20°c.

In a second method (P.D. Evans, personal communication), the homogenised adrenal medulla was centrifuged at 12,000 g for 12 min. After removing the fat, the supernatants were centrifuged at 100,000 g for 40 min. Ammonium sulphate was added and the fraction precipitating between 30 and 50% was dialysed against 0.01 M Tris HCl, pH 7.4 with frequent changes of buffer. The dialysed protein was concentrated to 5 cm^3 before elution on a Sphacryl S-200 column (100X 1.5 cm i.d.) previously equilibrated with 0.05 M Tris HCl, pH 7.4. The elution rate with Tris buffer was 5 cm^3 h^{-1} and 4 cm^3 fractions were assayed and pooled as previously described.

5.3 Results

The distribution of octopamine in selected tissues from adult and larval S. littoralis tissue is given in Table 19. The authenticity of the products formed for each type of tissue are given in Appendix 5. At least 90% of the radioactivity co-chromatographed with authentic synephrine for each tissue in three different solvent systems.

The standard curve was linear over the range 25 to 2000 pg. Blank values and therefore the ultimate sensitivity of the assay depended on the age of the SAM and some difficulty was found with stability and radiochemical purity of different batches. On storage, SAM decomposes to s-adenosyl-L-homocysteine an inhibitor of PNMT and this considerably reduced sensitivity.

The recovery of the internal standard from nervous tissue was greater than 90%. In non-nervous tissue, especially the haemolymph, recovery was sometimes reduced to between 50 and 60%.

5.4 Discussion

5.4.1 Octopamine in nervous tissue

The concentration of octopamine in nervous tissue (Table 19) is expressed as picomoles of octopamine per unit wet weight and per piece of tissue. Many authors have used the first method but Evans (1980a) emphasises there are drawbacks. He found the weight of adult locust nervous tissue increases with time from the final moult but octopamine content remains the same. In this study, tissue was sampled from insects of the same age, reducing variation between individuals. However, cell density may vary between insect species and comparison of the octopamine content on a unit per weight basis must be interpreted with caution.

Table 19. Octopamine content of S. Littoralis tissue

Sixth Instar Larvae	Mean tissue weight (µg) ± S.E.M.	n	pmole/Tissue ± S.E.M.	pg/mg wet weight of Tissue ± S.E.M.
Cerebral ganglion	109.1±6.2	(8)	1.51±0.59	2115.04±82.48
Sub-oesophageal ganglion	72.3±3.0	(8)	0.97±0.07	2051.07±150.14
Thoracic ganglia (3)	230.3±18.5	(8)	0.99±0.09	658.37±59.08
Abdominal ganglia (6)	436.5±29.5	(8)	1.04±0.11	363.76±38.31
Terminal ganglia	104.5±6.9	(8)	0.64±0.64	942.28±121.27
Salivary glands(per pair)	3710.0±390.0	(6)	0.40±0.03	16.33±1.19
Malpighian tubules	6677.0±533.0	(6)	1.00±0.09	22.92±1.99
Fat body	-	(6)	-	7.82±1.38
Muscle	-	(6)	-	27.36±5.96
Testis	-	(6)	-	104.36±17.75
Haemolymph	-	(6)	-	18.59±3.41
ADULT TISSUE				
Cerebral ganglion	383.5±36.40	(6)	5.78±0.08	2310.53±31.94
Optic lobes	779.0±42.00	(6)	5.24±0.32	1029.44±61.48
CNS (minus cerebral ganglion)	887.2±103.00	(6)	0.90±0.15	155.60±26.18
Spermiduct	-	(6)	-	111.63±12.39
Oviduct	-	(6)	-	188.48±23.97

Evidence is accumulating that D(-) octopamine is the naturally occurring isomer. It has been detected in locust haemolymph (Goosey & Candy, 1980) and the cerebral ganglion of Mamestra configurata (Starratt & Bodnaryk, 1981). In this study, DL-octopamine was used for the standard curves. The rates of methylation of D(-) by PNMT was five times the L(+) isomer giving 40-50% higher values (Starratt & Bodnaryk, 1981). To obtain the absolute amount of octopamine in each tissue a correction factor must be applied. However, DL-octopamine has been used in the majority of studies and synthesis of the D(-) octopamine, does not seem justified.

Despite these qualifications, the octopamine content of S. littoralis tissue appeared similar to other insects being highly localised in the ventral nerve cord, with the highest concentration in the cerebral ganglion of both larvae and adults. Values calculated from Bodnaryk (1980) for the cerebral ganglion (2800 pg/mg) and pair of optic lobes (4238 pg/mg) of another noctuid Mamestra confugurata compared with 2310 and 1029 pg/mg in the corresponding tissues of S. littoralis. Bodnaryk (1980) did not weigh the tissue actually used in the assay and calculated wet weight from dry weight by assuming 80% of the brain was water. In the only other report of octopamine in Lepidoptera tissue, Robertson and Jurio (1976) report the octopamine content of Manduca sexta thoracic ganglia to be 300 pg/mg. This compares to a value of 119 pg/mg for a single thoracic ganglion from S. littoralis. Some of the octopamine in thoracic and abdominal ganglia may be located in the dorsal median group of cells which stained with Neutral red (section 4.3.1). Isolated dorsal median somata have been shown to contain octopamine in locusts (Evans & O'Shea, 1978) and cockroaches (Dymond and Evans, 1979). No octopamine could be detected in soma which failed to stain with Neutral red. It is more difficult to locate octopamine-containing cells in the cerebral ganglia although possible

areas are those which do not contain FIF. In locusts and cockroaches the globuli cells in the calyx of the mushroom bodies do not fluoresce when treated with formaldehyde but do stain with Neutral red and contain considerable amounts of octopamine (Dymond & Evans, 1979).

The results of Neutral red staining of adult cerebral ganglia in the present work were disappointing. Discretely stained areas could not be identified as in the cockroach and locust, although 383 pM of octopamine were detected by radioassay. It may be significant that mushroom bodies are thought to receive multimodal responses to gut, taste, scent and mechanical stimulation. They may function as association centres where one sensory modality can modulate the response characteristics of a particular neurone to an imput from a second sensory modality (Evans, 1980a). It is interesting to speculate whether some of the aberrant behaviour is caused by formamidines interacting with the functions of octopamine in the mushroom bodies.

5.4.2 Octopamine in non-neuronal tissue

The presence of octopamine in the haemolymph of S. littoralis may be significant and support the proposed neurohormonal role for octopamine and possible interaction by formamidines. Octopamine is present in the haemolymph of Locusta migratoria at 10 pg/mg, 65 nM (David & Lafon-Cazal, 1979) and 33 pg/µl, 170 nM (Orchard et al., 1981). D(-) octopamine occurs in Schistocerca americana at a level of 5 pg/µl, 33 nM (Gooosey & Candy, 1980). This compares with a value of 19 pg/µl, 124 nM in S. littoralis larvae.

Goosey and Candy (1980) stated that radioenzymic assay allows good estimates of the octopamine content of nervous tissue where the concentration of this amine is relatively high. They found in locust haemolymph methylation of other compounds gave erroneously high results but this could be reduced by selectively isolating the radioactive

synephrine by co-crystalization with carrier synephrine. However, their assay was not carried out under optimal conditions but with an unusually long incubation period (1 h). Under these conditions, the synephrine formed may undergo further methylation to dimethyl-octopamine. With the shorter incubation time and TLC of the reaction products used in this and other studies, co-crystalisation with synephrine is unnecessary.

Recently, the conditions in which haemolymph octopamine levels change have been defined. During locust flight, there was a rapid increase from 33 to 173 nM within 10 minutes, declining almost to resting levels after 60 minutes. A similar concentration (300 nM) of octopamine stimulated glucose oxidation in isolated working flight muscles of the locust (Candy, 1978). Five hundred nM of this amine activated cockroach nerve cord glycogen phosphorylase (Robertson & Steele, 1972). Downer (1979a) believes octopamine mediates excitation induced hypertrehalosemia, 500 nM stimulating trehalose release from cockroach fat body (Downer, 1979b) as well as elevating cAMP (Gole & Downer, 1979).

A similar rise in octopamine and lipid levels was observed in the locust; 200 nM stimulated isolated locust fat body to release lipid (Orchard, 1972). Circulating levels of octopamine appear sufficient to stimulate the release of both lipid and carbohydrate. Octopamine in S. littoralis haemolymph may have an analogous role which may be mimicked by formamidines.

Octopamine was also detected in salivary glands, Malpighian tubules, fat body and muscle in S. littoralis (Table 19). Higher concentrations were present in adult oviduct and spermuduct where similar high values have been found in the locust (David & Lafon-Cazal, 1979). The physiological significance is unknown.

Chapter 6

EFFECTS OF FORMAMIDINES ON CARBOHYDRATE AND LIPID METABOLISM IN S. LITTORALIS

6.1 Introduction

The primary site of action for most insecticides is the nervous system. Closely linked is the neuroendocrine system and a number of insecticides cause the release of one or more neurohormones. For example, during poisoning representatives of various insecticide classes are thought to release hyperglycemic and adipokinetic hormones from the locust (Samaranayaka, 1974); diuretic hormone and plasticising factor from Rhodnius prolixus (Maddrell & Casida, 1972) and hyperglycemic factor from P. americana (Granett & Leeling, 1972).

These studies concentrated on demonstrating insecticide induced neurohormone release during the paralysis stage which occurs many hours after the initial poisoning. For example, Samaranayaka (1974) investigated the effects of lindane, a chlorinated hydrocarbon which acts at cholinergic synapses causing multiple discharges (Osborne, 1980). During the paralysis stage, about 15 h after treatment, fat body glycogen and haemolymph trehalose were significantly reduced but lipid levels increased. The author suggested the changes were caused by release of AKH and hyperglycemic hormone. At the start of this study, the effect of formamidines on the insect neuroendocrine system had not been investigated. Unlike conventional insecticides, inducing abnormal behaviour such as hyperactivity, which leads to erratic mating, reduced feeding and dispersal from the host are an important part of insect control by formamidines. Possible targets for formamidines causing abnormal behaviour are those systems concerned with stress and arousal.

A number of sub-lethal effects of formamidines have been mimicked by octopamine such as anorexia in cockroaches (Beeman & Matsumura, 1978b) and modulation of electrical activity in fourth instar M. sexta abdominal ganglia. Octopamine has been shown to have an effect on insect fat body (Downer, 1979a), nerve cord (Robertson & Steele, 1972) and muscle (Candy, 1978). The close structural similarity between formamidines and phenolamines (Section 1.5) suggested this group of insecticides might mimic directly the putative neurohormonal actions of octopamine rather than induce the release of one or more neurohormones. If true, this would be significant since no other insecticide group has previously been reported to interfere in this way with the biogenic amine system. The principle organic compounds, carbohydrates and lipid were therefore examined in sixth instar larvae following treatment with formamidines.

6.2 Materials and Methods

6.2.1 Determination of haemolymph lipid

Healthy sixth instar larvae, ranging from 600-800 mg, were held individually in Petri dishes, without food, under the same environmental conditions as described for the main culture (section 2.1.1). They were isolated at least two hours before sampling the haemolymph. Experiments were carried out during the first quarter of the light cycle.

Lipids were analysed by the sulphophosphovanillin reaction, modified for insect tissue by Stone and Mordue (1980).

Haemolymph was collected in glass micropipettes from a small puncture made in the proleg of the larvae (5 µl). The haemolymph was blown directly into 100 µl of chloroform/methanol (2:3, v/v) to precipitate protein. A sample was then added to 500 µl of concentrated sulphuric acid, heated to 100°C for 10 min in a Grant BT3 heating

block before cooling in ice. One hundred µl were added to 1 cm^3 of vanillin reagent (13 mM vanillin in 14 M orthophosphoric acid), mixed immediately and allowed to stand for 30 min. The optical density was read at 546 nm within 10 min, against a reagent blank processed in the same way, in a Beckman DBGT dual beam spectrophotometer.

Standard curves were constructed using cholesterol, with a range of 10-200 µg, five replicates at each concentration. There was a linear relationship between optical density and lipid concentration over this range. Lines were fitted by linear regression analysis.

All glassware was cleaned with chromic acid and solvents redistilled to avoid contamination.

6.2.2 Determination of carbohydrates

Carbohydrates were analysed by a modification of the method of Roe (1955). Trehalose, glucose and glycogen all give a positive reaction with the anthrone reagent.

Haemolymph (2 µl) was blown directly into 1 cm^3 of freshly prepared anthrone reagent (0.05% anthrone wt/vol, 1% thiourea wt/vol in 72.6% v/v sulphuric acid). After heating for 10 min at 100°C, the solution was cooled in ice before immediately measuring the optical density at 650 nm. The reagent blank consisted of 2 µl distilled water in 1 cm^3 anthrone reagent.

A calibration curve was constructed (using trehalose as the standard) in a similar way to the lipid standards. There was a linear relationship over the range 5-100 mg and the line was fitted by linear regression analysis.

6.2.3 Acid-alkali stable anthrone positive material (Trehalose)

Insect haemolymph is characterised by high concentrations of the non-reducing disaccharide, trehalose and a small proportion of reducing

sugars, principally glucose. Treatment of the sample with alkali destroys the glucose component and is considered to be a measure of trehalose present in the haemolymph (Gilroy, 1975).

The proportion present in sixth instar haemolymph was estimated by collecting duplicate samples (5 µl) in glass micropipettes. One sample from each pair was randomly assigned to measurement of total anthrone positive material as described in the previous section. Acid/alkali stable material was measured by heating the second sample with 100 µl of 0.75 M sulphuric acid for 10 min. at 100°C. After cooling, 100 µl of 5 M sodium hydroxide were added, heated for a further 10 min. at 100°C, cooled and then analysed in the anthrone assay. A standard curve was constructed using trehalose processed in the same way.

6.2.4 Determination of glycogen

Isolation of glycogen

Glycogen was isolated by the method of Carroll et al. (1956). The CNS of sixth instar larvae were rapidly dissected (in about 4 min) and immersed in pre-weighed microtubes containing 100 µl of potassium hydroxide (30% wt/vol). After weighing, tissue was digested by heating for 30 min at 100°C. Glycogen was precipitated by adding 120 µl of 95% ethanol and 20 µl of a saturated solution of sodium sulphate and leaving for 24 hours at 4°C. Following centrifugation at 10,000 g for 10 min, the supernatant was discarded and the pellet resuspended in 100 µl of distilled water.

Fat body was frozen immediately after removal on aluminium foil and lyophilised. The weighed tissue was disolved in 2 cm^3 of potassium hydroxide at 100°C. Five hundred µl samples were processed in the same way as the CNS.

Estimation of glycogen

Two techniques were evaluated; the reaction of glycogen with phenol/sulphuric acid (Montgomery, 1957) and the anthrone reagent (Carroll et al., 1956). Neither method is specific for glycogen. The former used reagents which were very stable, but was not as sensitive as the anthrone reagent. Glycogen was therefore estimated by the method already described for haemolymph carbohydrate.

6.2.5 Haemolymph volume

Haemolymph volume was monitored using the radiolabelled inulin method (see for example, Wharton et al., 1965). Inulin (^{14}C)-carboxylic acid (specific activity of a typical batch, 222 Mbq/mmol, 6 mCi/mmol) was diluted with saline to give approximately 77,000 dpm/µl. Five µl were injected into sixth instar larvae (weight range 600-800 mg), using a 33 needle gauge and the wound sealed with beeswax. Care was taken to avoid puncturing the gut. The insects were isolated in Petri dishes, without food and water and under the same conditions as the main culture.

Preliminary experiments indicated the inulin was dispersed within two hours. After leaving the larvae undisturbed for this length of time at 25°C and 70% R.H., solutions of formamidines were injected in saline using saline only for controls. At the same time a 5 µl sample was removed, digested in 0.1 cm^3 hyamine hydroxide, neutralised with 0.01 cm^3 acetic acid to reduce chemoluminescence and counted.

A series of 5 µl samples were removed at intervals after time 0 and treated in the same way.

Loss of inulin through excretion was determined by collecting frass and from absorption by counting the gut and its contents. Numerous investigations have failed to find evidence of inulin metabolism in a related species (see Levenbook, 1958) and this was not investigated

further.

Calculation of the results

Haemolymph volume was calculated from the following formula:

$$V = \frac{V_s (C_o - xC_o)}{C_b} - Vi$$

V = haemolymph volume (µl).

V_s = volume of sample (µl).

V_i = volume of inulin solution injected (µl).

C_o = DPM of inulin solution injected.

C_b = DPM of haemolymph sample.

X = correction factor due to excretion.

Haemolymph volumes estimated by this method were compared with values obtained by puncturing the prolegs of larvae onto pre-weighed filter paper and weighing the exuded haemolymph.

6.3 Results

There was considerable variation between haemolymph carbohydrate and lipid levels in sixth instar larvae. There was also considerable differences in the magnitude of the response to formamidines which greatly hindered progress in comparing control and treated insects.

Such variation in organic constituents of the haemolymph has been reported by many other authors. Carbohydrate and lipid levels are influenced by factors such as handling (Mathews & Downer, 1974; Orchard et al., 1981), feeding (Green, 1964) and photoperiod (Turner & Acree, 1967).

These factors were controlled, insects being reared on a defined artificial diet under constant conditions of temperature, lighting and

humidity. Experiments were carried out in the first quarter of the light cycle. Care was taken to handle larvae as little as possible. Viruses are known to cause metabolic disturbances in insects. Only (apparently) healthy insects were used. Insects do not appear to regulate carbohydrate and lipids within such narrow limits as the vertebrates.

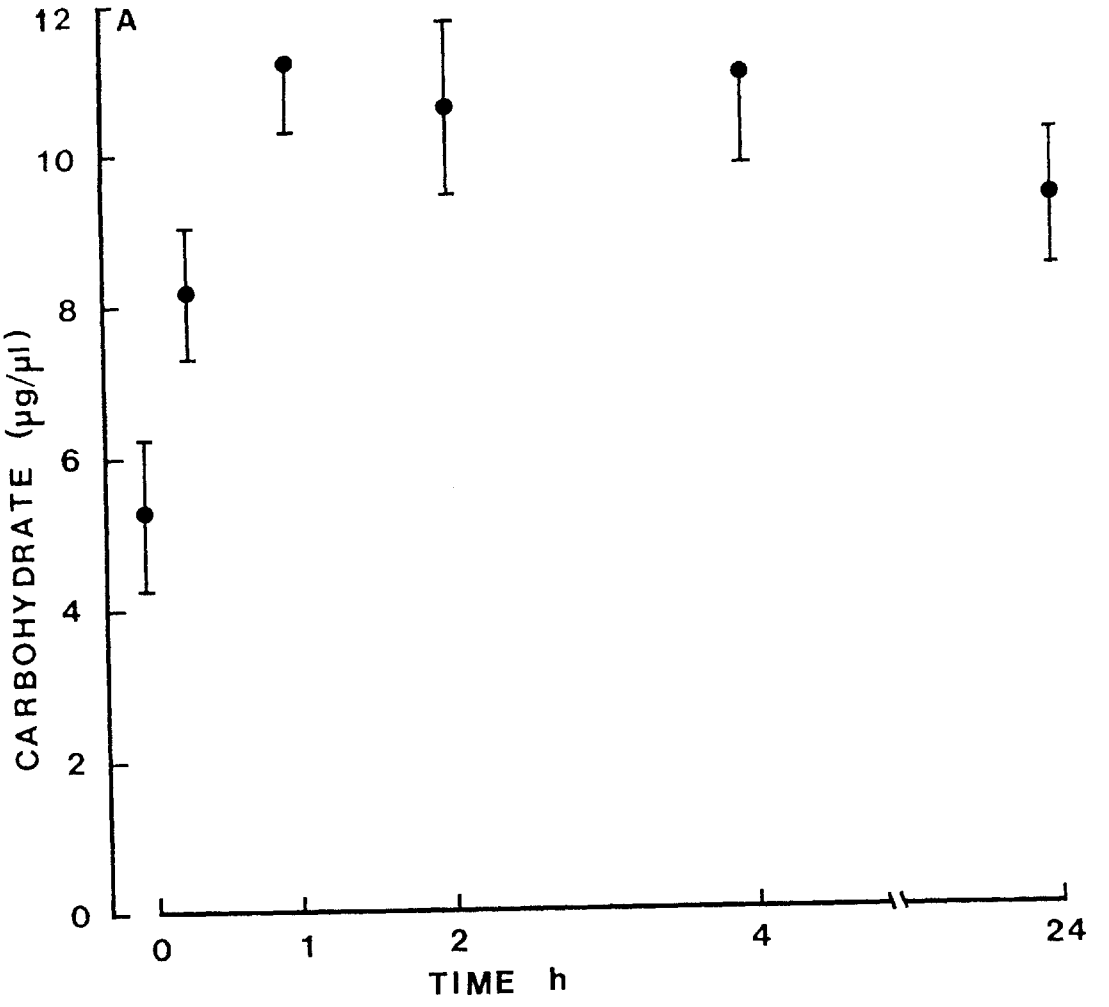
Trehalose was found to be the major component of S. littoralis haemolymph. There was 6.2 ± 1.5 µg/µl (n = 30) of total anthrone positive material compared to 5.9 ± 1.8 µg/µl (n = 30) acid/alkali stable material or 95% of the total.

CDM.HCl (LD_{10}) injected into sixth instar larvae significantly increased haemolymph carbohydrate and lipid (Figs. 7 & 8). This response occurred within 15 min of injection and levels remained elevated for at least two hours. During this period the insects became hyperactive. In control insects, there was a decline or no significant change. Hyperglycemia persisted for up to 24 h. It should be emphasised the response was variable, some individuals showed doubling of haemolymph levels, others only a small increase and it was difficult to establish a consistent trend after two hours.

The nature of the increase in haemolymph carbohydrate was investigated further. Injection of 10 µl 10^{-4}M octopamine (Fig. 9) caused a significant increase, but unlike CDM.HCl, levels were declining to resting values within one hour. Injection of 10 µl 10^{-4} M synephrine however had no significant effect.

Injection of 10 µl 10^{-4} M phentolamine mesylate (a gift from Ciba) apparently reduced hyperglycemia induced by LD_{10} CDM.HCl. Insects ligatured some hours before injection of CDM.HCl still showed a significant elevation in carbohydrate but this was delayed for some time (Fig. 10).

Fig. 7. Carbohydrate levels in the haemolymph of sixth instar larvae at various times following injection at time 0 of (A) LD_{10} CDM.HCl (85 µg/g of insect) and (B) saline only (n = 10 ± S.E.M.). CDM.HCl significantly increased carbohydrate compared to the resting level at time 0 ($p \leq 0.05$, t-test). There was no significant increase in the controls.



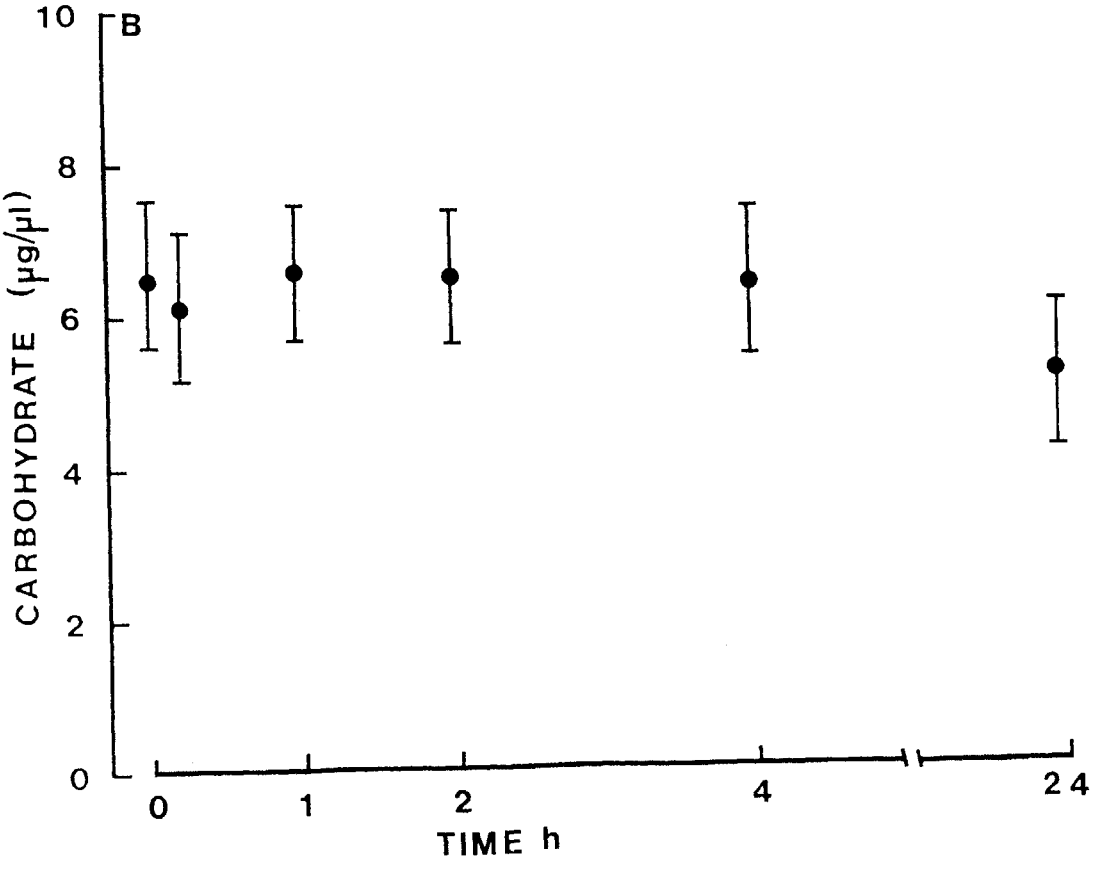
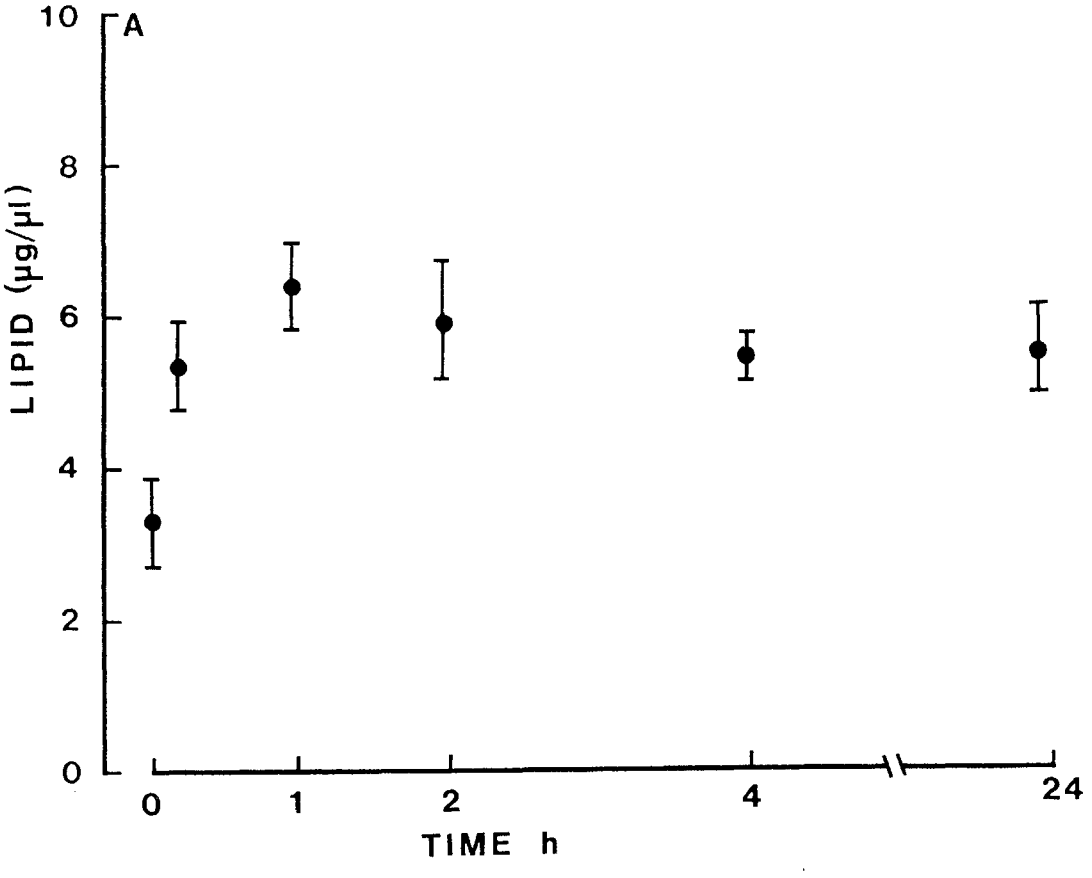


Fig. 8. Lipid levels in the haemolymph of sixth instar larvae at various times following injection at time 0 of (A) LD_{10} CDM.HCl (85 µg/g of insect) and (B) saline only (n = 10 ± S.E.M.). CDM.HCl significantly increased lipid compared to the resting level at time 0 ($p \leq 0.05$, t-test). There was no significant increase in controls.



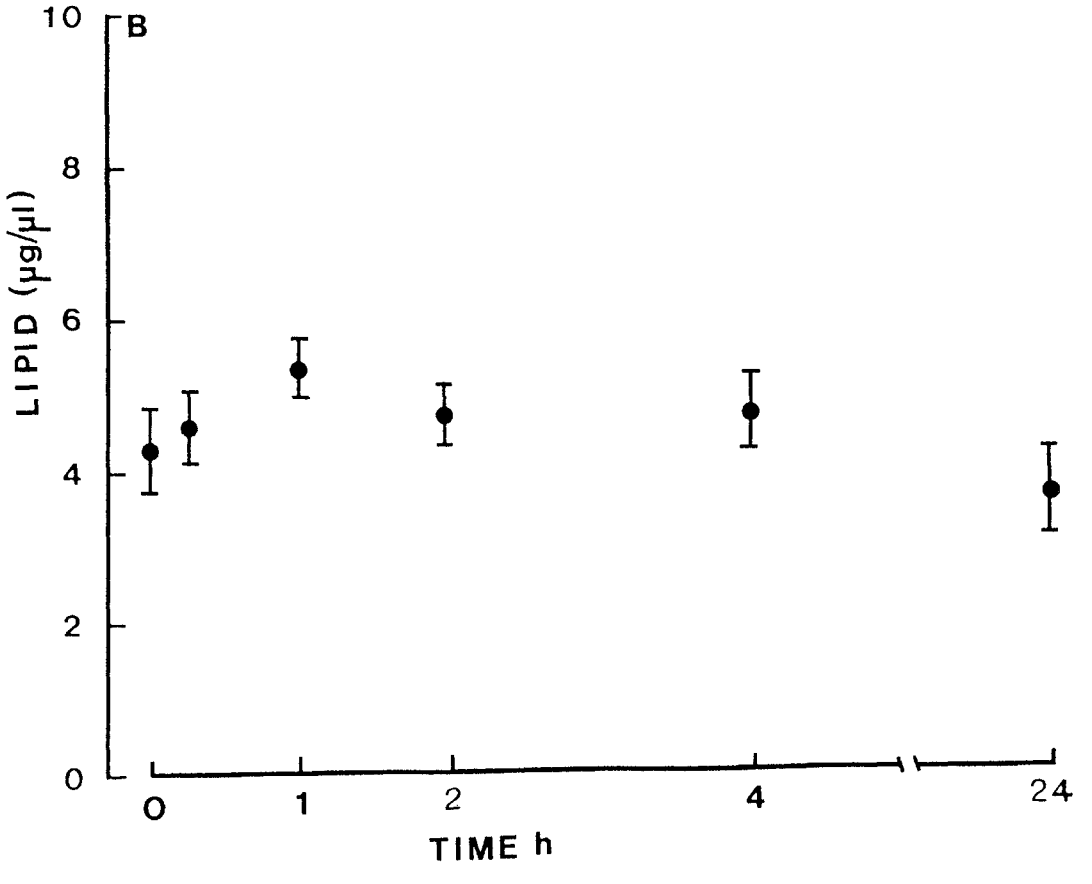
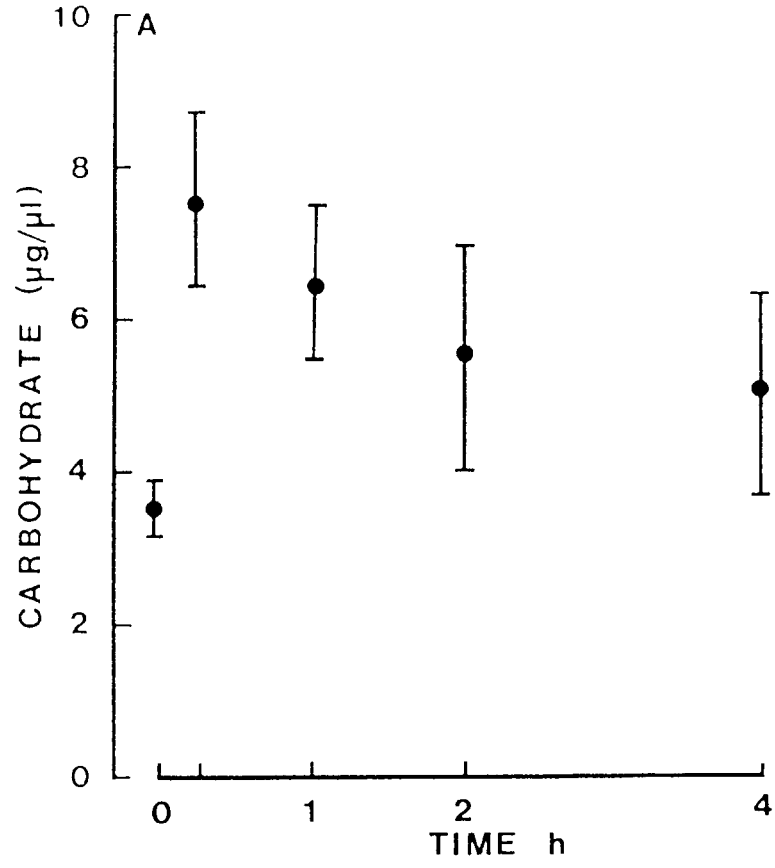


Fig. 9. Carbohydrate levels in the haemolymph of sixth instar larvae at various times following injection at time 0 of 10 µl 10^{-4}M octopamine (A) and 10^{-4}M synephrine (B) (n = 10 ± S.E.M.). Octopamine significantly increased carbohydrate after 15 m and 1 h ($p \leq 0.05$, t-test). The increase after injecting synephrine was not significant.



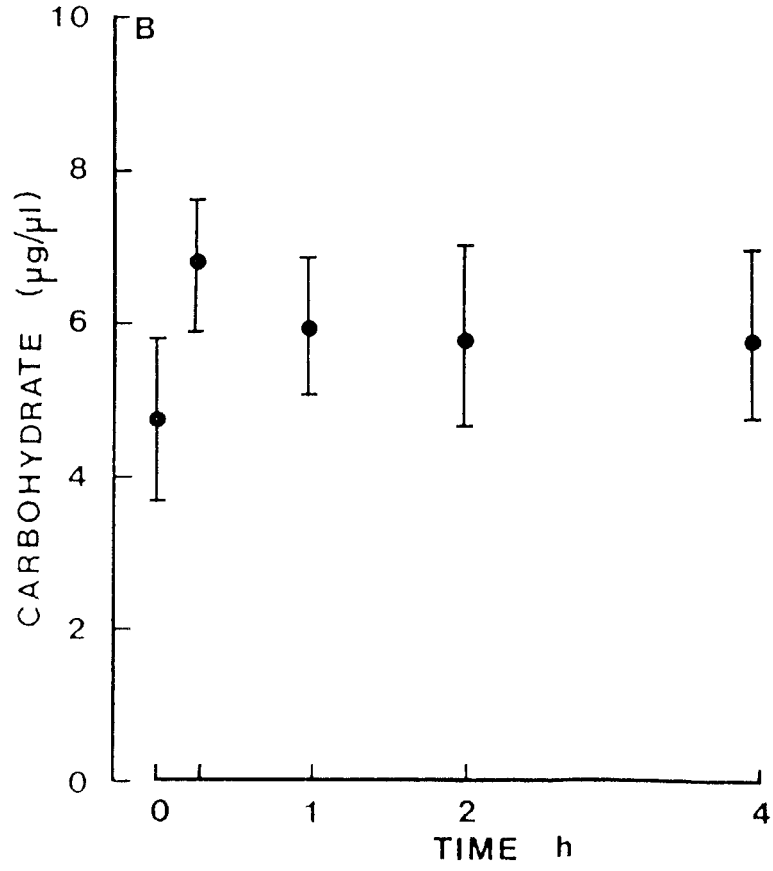
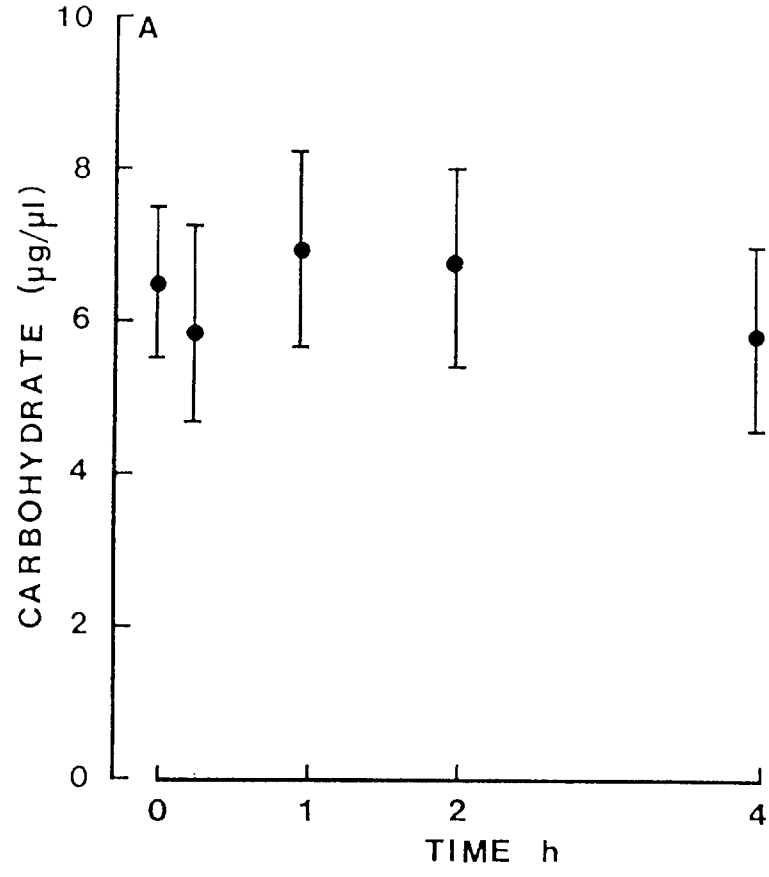


Fig. 10. Carbohydrate levels in the haemolymph of sixth instar larvae at various times following injection of LD_{10} CDM.HCl previously treated with 10^{-4} M phentolamine (A) or neck ligated (B) (n = 10 ± S.E.M.). Carbohydrate levels in neck ligated insects significantly increased after 1 h ($p < 0.05$, t-test) but there was no significant change in insects treated with phentolamine.



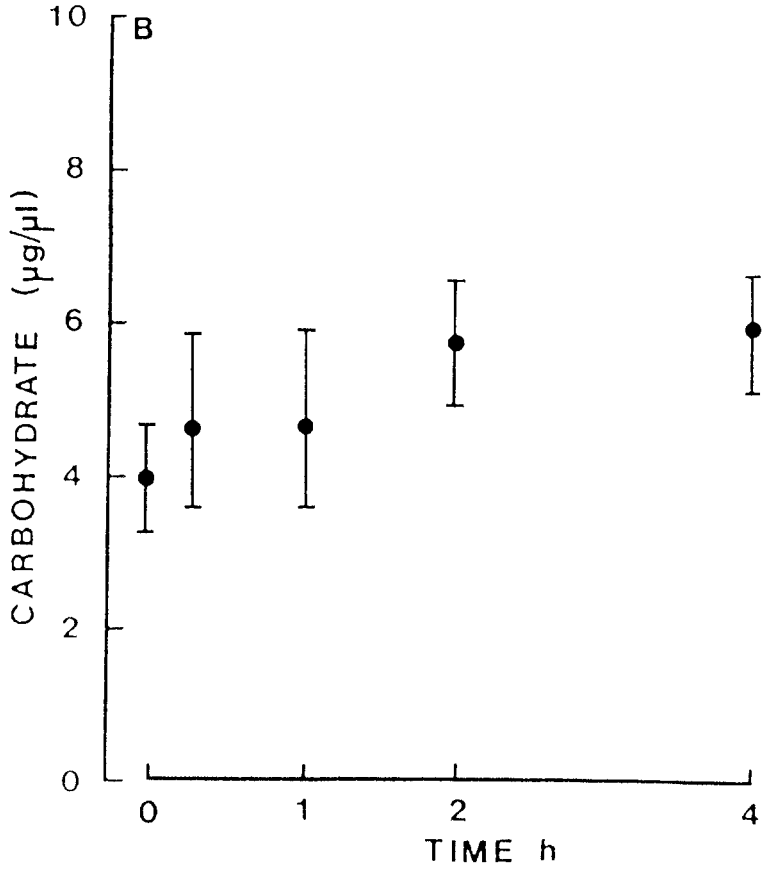


Table 20a. Effect of injecting an LD_{10} of CDM.HCl on the in vivo glycogen content of sixth instar ventral nerve cord and fat body

Ventral nerve cord

	n	µg/mg wet weight of ventral nerve cord ± S.E.M.	Mann-Whitney U-test
Control	9	5.37 ± 0.39	p = 0.0016
CDM.HCl	12	3.35 ± 0.23	

Fat body

	n	µg/ml dry weight of fat body ± S.E.M.	Mann-Whitney U-test
Control	10	46.14 ± 6.96	p = 0.0062
CDM.HCl	12	21.94 ± 2.49	

Table 20b. Change in haemolymph volume following injection of formamidines (LD_{10}) after 2, 6 and 24 h

Time		Control		CDM.HCl (LD_{10})			Amitraz (LD_{10})	
h	n	µl	% change	n	µl	% change	n	µl	% change
0	7	404±30	-10	9	265±22	-23	6	305±19	-17
2		363±30			205±24			254±37	
0	7	433±44	-14	8	371±50	-12	6	483±80	-16
6		374±56			328±28			404±55	
0	7	433±44	-17	8	371±50	-20	6	483±80	-20
24		358±54			296±29			388±51	

No significant difference between control and treated insects after 2, 6 or 24 hours (Mann-Whitney U-test).

CDM.HCl significantly reduced fat body glycogen after 2 h suggesting this was the source of increased haemolymph carbohydrate. Glycogen stores were also significantly depleted in the ventral nerve cord (Table 20a).

Sub-lethal doses of some insecticides cause excessive water loss mainly by increased evaporation due to a failure to close spiracular valves. Other significant losses occur by transport of fluid across the gut wall and regurgitation (Samaranayaka, 1974, 1977).

Increases in carbohydrate and lipid might result from a reduction in haemolymph volume. This possibility was tested by injecting formamidines and monitoring haemolymph volume at 2, 6 and 24 h after injection.

Table 20b indicates there was a gradual decline in haemolymph volume but there was no significant difference between control and treated larvae. At this dose, formamidines do not have any effect on the water regulation in this insect.

6.4 Discussion

6.4.1 Effect of formamidines on carbohydrates

The composition of insect haemolymph reflects the balance between the synthesis of organic compounds from dietry precursors and metabolism by tissues. In locusts, the most extensively studied insect, lipids are regulated by a peptide hyperlipemic neurohormone (AKH) released from the glandular lobe of the corpora cardiaca. Release is controlled by axons from NCCII (Stone & Mordue, 1980). This action is opposed by a hypolipemic factor, also under NCCII control but released from the storage lobe (Orchard & Loughton, 1980).

Carbohydrates are regulated by a peptide hyperglycemic factor (Loughton & Orchard, 1981) released after stimulation of NCCII which these authors believe to be similar to AKHII of Carlsen et al. (1979)

which also has hyperlipemic activity similar to AKH. A hypoglycemic factor has also been proposed in the brain neurosecretory cells of flies (Candy, 1981).

In Lepidoptera, neurohormonal regulation is less well understood. Since Steele (1961) first demonstrated the presence of a hyperglycemic factor in the cockroach, similar hyperglycemic effects have been demonstrated in other insects, including Lepidoptera, although not all insects show this response (Candy, 1981). Tager et al. (1976) found glucagon and insulin-like immunoreactive material in the corpora cardiaca/corpora allata complex in M. sexta. Kramer et al. (1980) have found components in the haemolymph of this moth which react to antibodies for insulin. Whether these results are significant is unknown. Immunochemical studies can give misleading results when they are the only criteria used. Hyperlipemic factor has been detected in corpora cardiaca extracts from M. sexta (Candy, 1981).

In addition to regulation by peptide neurohormones, there is growing evidence that octopamine is released into the haemolymph in response to stress, increasing carbohydrate and lipid levels in an analogous way to vertebrates (section 5.4.2) (Orchard, 1982). It should be emphasised that substrate regulation is a dynamic and complex process with many factors interacting in response to changing physiological requirements.

The polysaccharide, glycogen, synthesised from dietry glucose is the major long-term carbohydrate store in fat body, gut and flight muscles (Chippendale, 1978). S. littoralis haemolymph (this study) like most other insects is characterised by high concentrations of the disaccharide, trehalose, forming an intermediate store. It can readily be hydrolysed to glucose to provide energy (Steele, 1981).

An increase in haemolymph carbohydrate by stimulating glycogenolysis in the fat body occurs after 1 h. The EXIT response

described by Downer (1979a) (section 5.4.2) is characterised by a maximal response within 15 minutes following stress. The elevated haemolymph trehalose returned to resting levels within 1 h and is independent of the corpora cardiaca. In addition to mimicking the EXIT response (Downer, 1979b), octopamine increased trehalose production from glycogen in isolated fat body and elevated cAMP (Gole & Downer, 1979; Downer, 1980).

There is very little information concerning neurohormonal regulation of carbohydrate in Lepidoptera and nothing has been published about S. littoralis. Any conclusions about the action of formamidines can only be tentative. The haemolymph of S. littoralis contains octopamine (19 pg/µl) at concentrations similar to the resting levels of other insects. The conditions under which this level changes, has not been examined. It is also a disadvantage that by using the intact insect it is not possible to be certain of the target for the formamidines. Despite these objections, by analogy with the regulation systems in other insects, the time course for the elevation of trehalose levels suggests mimicry of the neurohormonal action of octopamine, rather than inducing the release of hyperglycemic hormone. Depletion of S. littoralis fat body glycogen suggests increased glycogenolysis is the source of the circulating trehalose.

The glycogen content of S. littoralis ventral nerve cord is within the range estimated for other insects; 1.98 ± 10.8 µg of glycogen/mg of tissue (Strang, 1981).

Injection of CDM.HCl significantly reduced glycogen levels in nerve cord by 38%. Robertson and Steele (1972) found 25 nmole of octopamine, injected into the cockroach, reduced ventral nerve cord glycogen by a similar amount (35%).

The physiological significance is unclear. Although glycogenolysis in the fat body leads to augmented trehalose levels in the blood (Steele,

1963) there is insufficient nerve cord glycogen to contribute to the haemolymph pool in S. littoralis. Nerve cord glycogen is deposited in the perineurium of the sheath, with smaller quantities in the glial cells (Strang, 1981).

Glycogen is a potential energy source; the cessation of spontaneous activity coincides with the reduction of glycogen to its minimum value. However, the conditions under which it is mobilised are not understood, especially when there are abundant exogenous carbohydrate substrates in the haemolymph.

6.4.2 Effect of formamidines on haemolymph lipids

In Orthoptera and Lepidoptera, the principle haemolymph lipid is diglyceride (bound to protein carriers) which are mobilised from fat body triglycerides by AKH (Beenakkers et al., 1981).

As with carbohydrates, octopamine has been implicated in excitation induced hyperlipemia (Orchard et al., 1981). Excitation of locusts caused by handling induced a rapid rise in both octopamine and lipid levels in the haemolymph. The response was detected within 2 min, was maximal after 15 and persisted for 1-2 h. The effect could be blocked by α-adrenergic blockers and potentiated by octopamine. Neck ligatured locusts still showed the response, suggesting it was independent of the corpora cardiaca, although the onset of rising lipid and octopamine levels was slower. Octopamine concentrations, similar to those measured in the haemolymph following stress, stimulated isolated fat body to release lipid. Evidence was presented showing that octopamine and AKH acted on different fat body receptors (Orchard, 1981).

Hyperlipemia in locusts was previously associated with prolonged flight. The flight muscles switch from trehalose to lipid utilization after 25 min of flight (Robinson & Goldsworthy, 1974). Although haemolymph

lipid has increased within minutes of flight initiation, AKH titres have not. Octopamine levels are at a maximum within 10 min (Goosey & Candy, 1980). This suggests the initial rise in lipid in the absence of AKH could be induced by octopamine. In addition, circulating octopamine may potentiate neurally evoked release of AKH by acting at the putative octopaminergic synapse between NCCII axons and the glandular lobe neurosecretory cells (Orchard & Loughton, 1981).

In a recent study, Singh et al. (1981) examined the effect of formamidines at this synapse. They found that incubation of locust corpora cardiaca in saline containing CDM and DCDM produced hyperlipemia when injected into adult locusts. This was blocked by α-adrenergic antagonists. CDM and DCDM potentiated the release of hyperglycemic hormone from the corpora cardiaca during electrical stimulation of NCCII. It was concluded that formamidines or their metabolites interacted with post synaptic octopamine receptors to release hyperlipemic hormone. These authors did not however inject formamidines into the whole insect and examine the effect on lipid levels.

It is impossible to determine the level at which the formamidines are acting in S. littoralis, since the experiments were performed on the intact insect. As with carbohydrates, insufficient information is available concerning the neurohormonal regulation of lipid in this insect. Some similarity to the locust might be expected since Lepidoptera also utilise lipid during flight. By analogy with the locust, if formamidines interact with octopamine receptors there are two potential targets: acting directly on the fat body to release the diglyceride or indirectly by inducing the release of hyperlipemic neurohormone via octopamine receptors in the corpora cardiaca. Biogenic amines have been implicated in the release of AKH. It may be significant that a non-fluorescent amine, such as octopamine, could be associated with

S. littoralis corpora cardiaca (section 7.4.3). It was impossible however to perform experiments on the isolated corpora cardiaca from the sixth instar because of the small size of the gland.

Release of octopamine in response to stress should be precise in quantity and time. Metabolic levels should return to normal when stressful stimulation ceases. If formamidines mimic this action, an inappropriate response particularly at critical points in the life cycle, will have profound physiological consequences, resulting in an unsuitable internal environment for normal functioning and subsequent modification of behaviour.

The evidence that one of the biochemical targets for the formamidines is the neurohormonal action of octopamine is at best circumstantial. To establish this idea, the response of isolated tissues such as fat body and corpora cardiaca to formamidines and phenolamines must be examined. Studies on intact insects yield only a limited amount of information. The dose dependence of such a response should be established. The response should be blocked by α-adrenergic but not β-adrenergic antagonists. However, there is a dilemma as to whether these effects should be examined in those species of insect in which the neurohormonal regulation is well understood, but are insensitive to the formamidines or target insects such as S. littoralis which show behavioural changes at sub-lethal doses, but their biochemistry is unknown.

Chapter 7

EFFECTS OF FORMAMIDINES ON THE METABOLISM OF BIOGENIC AMINES IN S. LITTORALIS TISSUE

7.1 Introduction

The radioenzymic assay used in this study is based on the method of Hayashi et al. (1977) and modified from Dewhurst et al. (1972). The assay involves the conversion of labelled amines by tissue homogenates to radioactive products which are extracted into an organic solvent. Originally developed as a sensitive assay for Monoamine oxidase (MAO) in vertebrates this method is non-specific, failing to distinguish between the activity of MAO and N-acetyltransferase.

7.2 Materials and Methods

7.2.1 Enzyme preparations

The CNS (including the cerebral ganglion), Malpighian tubules, fat body and salivary glands were dissected from sixth instar larvae S. littoralis and each tissue was weighed. Haemolymph was collected from a puncture made in a proleg.

Tissue was homogenised in 100 µl of 0.18 M KCl in a Jencon glass microhomogeniser at 2-4 °C. All further stages in the enzyme preparation were carried out at this temperature.

In other experiments, a series of homogenates from sixth and fourth instar tissues were tested for activity. These were compared with characterised sources of MAO from rat liver and the tick Boophilus microplus.

In preliminary experiments several procedures for preparing the enzyme were used in an attempt to concentrate enzymic activity (which was considerably lower in S. littoralis tissues than in the rat or tick).

A procedure for isolating mitochondria from the whole insect was also developed. All tissue was weighed and homogenised in a glass/Teflon Potter-Elveljem homogeniser at 2-4 °C with a known volume of 0.15 M KCl. In some experiments the product was further disrupted in an MSE 150 W ultrasonic disintegrator for specific periods of time at an amplitude of 12 KC, using a 9 mm diameter probe.

The homogenate was centrifuged at 500 g for 20 min. at 2 °C. The supernatant was removed and re-centrifuged at 39,000 g for 30 min. The pellet containing the mitochondria (and the majority of the enzymic activity) was resuspended in a known volume of 0.25 M phosphate buffer (pH 7.4).

7.2.2 Radioenzymic assay

A typical incubation mixture contained 50 µl of enzyme and 50 µl of 0.1 M potassium phosphate buffer, pH 7.4. Hayashi et al. (1977) found in locust nervous tissue that choline acetyltransferase competes with N-acetyltransferase for acetyl co-enzyme A. Acetylcholine and eserine were therefore added with the buffer to give a final concentration of 2 mM and 2.3 µM respectively.

The enzyme was pre-incubated for 10 min. with various concentrations of formamidines and MAO inhibitors in a shaking water bath at 25 °C.

The reaction was initiated by adding radiolabelled amine in 50 µl of phosphate buffer containing the appropriate unlabelled amine to give a final concentration of 1 mM. The following amines were used; [side chain-1-^{14}C]tyramine hydrochloride (50 mCi/mmol, 1.85 GBq/mmol), ^{3}H tryptamine hydrochloride (general tritium labelling, 1.3 Ci/mmol, 48.1 GBq/mmol) and DL-[3,5,^{3}H]octopamine hydrochloride, (37 Ci/mmol, 1.37 Tbq/mmol). The buffer also contained acetyl coenzyme A, final concentration 1 mM. The final assay volume was 150 µl.

Blanks consisting of the same reaction mixture but with denatured enzyme were included.

7.2.3 Solvent extraction

After incubating the mixture for 60 min. at 25 °C, the reaction was stopped by adding 500 µl of 2 M HCl. Two cm^3 of ethyl acetate was added to each tube, vortexed for 30 s and centrifuged at 10,000 g for 5 min. A 1.5 cm^3 portion was removed and washed with 1 cm^3 HCl. After repeating the mixing and centrifugation step, a 1 cm^3 sample was counted.

7.2.4 Double label radioenzymic assay

Enzyme preparations from selected tissue were also incubated in the presence of ^{14}C tyramine and β-[3-^{3}H] alanine (32 Ci/mmol, 1.18 Tbq/mmol).

The reaction was initiated by adding the radiolabelled compounds in 50 µl of phosphate buffer which also contained unlabelled tyramine and β-alanine to give a final concentration of 1 mM for each compound.

The assay conditions and extraction procedure were the same as described in section 7.2.2. The two isotopes were counted as outlined in section 2.3.2.

7.2.5 Thin layer chromatography

After the final wash in 2M HCl, the solvent from five replicates of each enzyme preparation was pooled, evaporated to a small volume under vacuum before eluting on silica gel (Merck) TLC plates backed by aluminium.

Separation of tryptamine and tyramine metabolic products was achieved using ethyl acetate and glacial acetic acid, 99:1 by volume (Evans et al., 1980). N-acetyltryptamine was synthesised from

tryptamine and acetic anhydride by the method of Beeman (1977). Octopamine metabolites were separated using butan-1-ol:acetic acid:water, 15:3:5 by volume (Maxwell et al., 1980).

The TLC plates were cut into strips. Standards were visualised under UV illumination after spraying with a 0.1 mg/cm^3 solution of fluorescamine in dry acetone or by exposure to iodine vapour.

Chromatogram strips containing radioactivity were divided into 0.5 cm strips, eluted and then counted in 5 cm^3 of Bray's scintillant.

7.3 Results

7.3.1 Effect of formamidines on biogenic amine metabolism in S. littoralis tissues in vitro

Octopamine metabolism by enzymic preparations from five types of larval tissue are presented in Table 22. Enzymic activity was low compared to values reported by Hayashi et al. (1977) for equivalent locust tissues. Activity was abolished above 35°C. Nishimura (1975) reported cockroach N-acetyltransferase was unstable above 30°C.

Generally, in nervous tissue, salivary glands and haemolymph, formamidines significantly inhibited enzymic activity but activity was not reduced in Malpighian tubules and fat body. Tranylcypromine, even at the high concentration used, failed to inhibit activity in any tissue, indicating MAO levels were low. Significant incubation of enzymic activity from Malpighian tubules by MAO inhibitors has been reported in other insects, including the study by Hayashi and co-workers (1977), but these authors were unable to detect p-hydroxymandelic acid, the oxidative product of octopamine.

In preliminary experiments, attempts to concentrate the enzymic activity by isolating mitochondria were not successful (Table 21). This procedure would be expected to increase the activity of enzymes such as MAO associated with mitochondria. CDM base and amitraz inhibited

Table 21. Effect of formamidines on the metabolism of ^{14}C-tyramine and ^{3}H tryptamine by a mitochondrial preparation from fourth instar S. littoralis.

Treatment	Concentration (M)	pmole of tyramine metabolised/ h/g of protein (n = 4 ± S.E.)	pmole of tryptamine metabolised/ h/g of protein (n = 4 ± S.E.)
Control		89.4 ± 1.5	1.0 ± 0.02
CDM	5×10^{-4}	82.6 ± 0.9^{+}	0.7 ± 0.01^{+}
CDM.HCl	5×10^{-3}	98.3 ± 2.3*	3.8 ± 0.3*
DCDM	5×10^{-4}	114.0 ± 1.9*	9.1 ± 0.3*
Amitraz	1.5×10^{-6}	76.0 ± 0.8*	-
Harmaline	1.5×10^{-4}	89.8 ± 2.9^{+}	0.8 ± 0.16^{+}

* Significant change in metabolism of labelled amine compared to the control at the 5% level.

+ No significant change. (Mann-Whitney U-test)

TABLE 22. Effect of formamidines on the metabolism of octopamine in sixth instar tissue of S. littoralis in vitro.

Treatment	Concentration (M)	pmole of Octopamine metabolised/h/g protein (n=4±S.E.M) CNS	Malpighian Tubules	Salivary gland	Haemolymph	Fat body
Control	-	1479.1±61.5	147.9±11.3	393.2±45.0	118.8±4.2	10.8±1.5
CDM	$1x10^{-6}$	972.9±219.8**	121.9±5.3+	212.5±1.1*	75.4±0.7**	6.9±0.3*
CDM.HCl	$1x10^{-6}$	1556.8±11.0+	153.0±3.2+	276.8±2.3*	82.3±2.9*	9.9±1.5+
DCDM	$1x10^{-6}$	1712.8±32.7+	145.5±6.7+	293.2±17.2**	88.2±3.4**	14.8±6.4+
Amitraz	$1.5x10^{-6}$	1052.0±87.9**	142.3±2.6+	315.6±33.2+	56.1±2.0**	10.2±0.4+
Tranylcypromine (MAO Inhibitor)	$1.5x10^{-4}$	1092.3±65.2+	153±1.0+	3961.1±95.8+	116.7±1.3+	11.1±1.5+

* Significant inhibition of enzyme activity at 5% level;

** Significant inhibition of enzyme activity at 1% level;

\+ No significant inhibition. (Mann-Whitney u-test).

this activity but CDM.HCl and DCDM unexpectedly increased the amount of radioactivity extracted into the solvent, apparently enhancing metabolism.

7.3.2 Thin layer chromatography of the labelled products

The presence of MAO in S. littoralis tissue would be expected to give the following final metabolic products: hydroxymandelic acid (octopamine), p-hydroxyphenyl acetic acid (tyramine) and indole-3-acetic acid (tryptamine). N-acetyltransferase would produce the corresponding N-acetylated amines.

Preliminary results from TLC of the reaction products were complex. Using labelled octopamine as the substrate at least 4 peaks were present in extracts from each tissue. One peak contained 10-11% of total radioactivity and co-chromatographed with DL-octopamine. Less than 1% of the radioactivity was associated with p-hydroxymandelic acid. This supports the result of MAO inhibitor study which indicated oxidative deamination activity was low in larval tissue. Two peaks eluted in front of p-hydroxymandelic acid containing 10-16% and 13-17% of the total radioactivity. Most activity was associated with a third peak eluting ahead of the octopamine standard (51-59%) which in the same solvent system corresponded to N-acetyl octopamine (Hayashi et al., 1977).

A similar number of peaks were detected with ^{14}C tyramine and ^{3}H tryptamine. A very small proportion (about 1%) was associated with unmetabolised amines. About 60% co-chromatographed with the N-acetylated amine. The remaining radioactivity was present in at least two other peaks, one eluting ahead of N-acetyltryptamine or N-acetyl-tyramine. Less than 1% eluted with oxidative deamination standards.

A major metabolite of tryptamine, tyramine and possibly octopamine appears to be N-acetylated. The identity of the other peaks is

unknown. Maxwell et al. (1980) in M. sexta found three products from labelled tyramine. Two were identified as N-acetyltyramine and β-alanyltyramine and the third was believed to be a sugar conjugate.

When S. littoralis tissue was incubated with ^{14}C tyramine and ^{3}H β-alanine only a small proportion of the ^{3}H label varied for each of the four tissues (CNS, fat body, Malpighian tubules and salivary glands) examined. TLC of the reaction products produced variable results and failed to provide any further evidence for the identity of the other peaks.

7.4. Discussion

7.4.1 Metabolism of biogenic amines

At vertebrate nerve terminals, a high affinity uptake mechanism removes amines from the synaptic cleft into the pre-synaptic membrane. Here they are inactivated by MAO associated with the mitochondria. A low affinity uptake mechanism is present in non-neuronal tissue where COMT is the degradative enzyme.

Insect biogenic amines serve a dual role in sclerotization and as chemical transmitters. Metabolism of amines and their derivatives has been extensively studied. Much less is known about inactivation of neurotransmitters and neurohormones. For example, no inactivating enzyme has been isolated or purified from insect tissue (Evans, 1980a).

During tanning biogenic amines are synthesised from tyrosine by haemolymph enzymes. The products may be protected from oxidation by conjugation with sulphates, phosphates and sugars. They are also bound to carrier proteins. It is not always clear whether the observed degradation of amines involves production of tanning intermediaries, metabolic detoxification of amines functioning as chemical transmitters or both (Evans, 1980a).

The presence of MAO has been demonstrated histochemically in specific areas of the insect brain, where the enzyme may have a local role in metabolism. Sensitive biochemical methods based on the breakdown of labelled amines failed to detect the enzyme, suggesting activity was very low (Evans & Fox, 1975; Dewhurst et al., 1972). Although there may be differences between insect species, evidence is accumulating significant activity is only present in Malpighian tubules (Hayashi et al., 1977) and the major route in nervous tissue is by N-acetylation.

The results from S. littoralis tissue support this. Oxidative deamination could not be detected even when mitochondria preparations were used in an attempt to concentrate the enzyme or in Malpighian tubules.

A degradative mechanism was present in each tissue examined, which in some cases was significantly inhibited by formamidines. The identity of the principle metabolites is uncertain but some products may result from N-acetyltransferase activity. In the locust, N-acetylation was the major route for octopamine metabolism in Malpighian tubules, salivary glands, fat body and haemolymph (Hayashi et al., 1977). In the only study on Lepidoptera, activity was detected in nervous tissue, tracheae and connective tissue with tyramine as the substrate (Moore et al., 1978; Maxwell et al., 1980). The widespread distribution of N-acetyltransferase makes this enzyme ideally suited as an inactivating mechanism for biogenic amines released into haemolymph and subsequently taken up by a range of tissues in an analogous way to vertebrate COMT. The enzyme is not simply to produce tanning intermediaries since it persists in adults when the tanning process has finished (Evans, 1980a).

Formamidines have been reported to inhibit locust (Nishimura et al., 1975) and cockroach (Beeman, 1977) N-acetyltransferase.

Although this action would contribute to formamidine toxicity in S. littoralis given the low levels of enzyme activity and the failure to inhibit activity in all tissues, it is unlikely to play a major role.

A second major pathway for tyramine metabolism has been reported in Lepidoptera which involves conjugation of the amino group of tyramine with β-alanine (Maxwell et al., 1980). Conjugates of β-alanine with tyrosine and octopamine have been found in arthropods (Levenbook et al., 1969; Kennedy, 1977). It is not yet known whether this is a functional system for inactivating biogenic amines or converting tanning intermediaries to storage forms (Evans, 1980a). Moore et al. (1980) believe the identity of a third metabolite is a sugar conjugate of N-acetyltyramine. A similar mechanism could exist in S. littoralis tissue and could account for two of the unknown compounds revealed by TLC.

SUMMARY

(1) Some of the behavioural, biochemical and physiological effects of formamidines and in particular chlordimeform (CDM) have been studied in S. littoralis.

(2) The toxicity of formamidines to S. littoralis is complex with lethal and sub-lethal effects, particularly at critical points in the life cycle.

(3) The toxicity of CDM base and CDM.HCl decreased from the fourth to sixth instars, but sensitivity was regained in adults. Amitraz was toxic to adults only.

(4) There was a dramatic and unexpected difference between the toxicity of CDM.HCl and CDM base to adult S. littoralis and a related noctuid H. virescens. This could not be explained by differences in penetration. The trend was reversed in the larvae, the base being the most toxic.

(5) CDM acts as a stomach and contact poison. Topical application was more effective than ingestion in killing larvae.

(6) DCDM was the only metabolite of CDM to show biological activity to S. littoralis and H. virescens but was much less toxic than the parent compound.

(7) Application of piperonyl butoxide, an inhibitor of microsomal mixed function oxidases synergised CDM, DCDM and amitraz in adult H. virescens. Piperonyl butoxide slightly antagonised CDM in fourth instar S. littoralis. In other organisms such as ticks, CDM and amitraz are not directly toxic but must be converted to N-monomethyl-formamidines such as DCDM. This is not true for H. virescens and possibly for Lepidoptera in general: the parent compounds are the actual toxicants.

(8) Sub-lethal concentrations of CDM significantly reduced fecundity in adult and feeding in larvae.

(9) Companion studies have attempted to find a biochemical and physiological explanation of this behaviour modification. The interaction of formamidines with biogenic amines and in particular octopamine has been investigated.

(10) The distribution of octopamine has been surveyed in larval and adult moths using histochemical techniques and a radioenzymic assay.

(11) The dye Neutral red which stains aminergic cells, revealed a group of cells in the sixth instar thoracic and abdominal ganglia of the ventral nerve cord. Some of these cells may correspond to dorsal median cells of other insects, one of which has been shown to be octopaminergic in the locust.

(12) Cell bodies were also detected in the frontal ganglion of the somatogastric nervous system and the corpora cardiaca.

Formaldehyde induced fluorescence (which localises primary and secondary monoamines but not phenolamines such as octopamine) was detected in cell bodies from the cerebral ganglion of S. littoralis, but could not be detected in cell bodies staining with Neutral red in the ventral nerve cord, suggesting they contained a non-fluorogenic amine, such as octopamine.

Octopamine, estimated by a sensitive and specific radioenzymic assay, was highly localised in the cerebral ganglion and ventral nerve cord of adults and larvae. Concentrations were similar to other insects.

(13) Octopamine was also present in non-neuronal tissue. The presence of octopamine in the haemolymph may indicate a neurohormonal role.

(14) In S. littoralis the biochemical target does not appear to involve inhibition of amine degradative enzymes such as Monoamine oxidase as has been suggested in some other species.

Degradative enzymic activity was found in each tissue examined. However, Monoamine oxidase could not be detected in S. littoralis tissue. Analysis of the reaction products showed amine catabolism in S. littoralis is complex, involving more than one pathway. There was some evidence for N-acetylation and possibly for conjugation with amino acids.

(15) In some tissues, formamidines significantly inhibited enzymic activity. This may contribute to formamidine toxicity in S. littoralis but given the low levels of activity and failure to inhibit amine metabolism in all tissues, it is unlikely to play a major role.

(16) Analysis of the principle classes of organic compounds in selected tissues from sixth instar larvae showed sub-lethal doses of CDM.HCl significantly elevated carbohydrate and lipid levels. Fat body and ventral nerve cord glycogen were significantly reduced.

(17) It is possible that one of the biochemical targets of formamidines is to mimic the neurohormonal action of octopamine which may be released under stressful conditions, stimulating carbohydrate and lipid metabolism, and increasing the amount of energy available for immediate use: the fight or flight response in insects.

(18) Release of octopamine should be precise in quantity and time with metabolic levels rapidly returning to normal when stressful stimulation ceases. An inappropriate response induced by formamidines, particularly at critical points in the life cycle, will have profound physiological consequences leading to an unsuitable internal environment for normal functioning.

APPENDIX 1 Preparation of artificial diet for rearing S. littoralis

Potassium hydroxide (6.25 cm^3) was added to 400 cm^3 of sterile distilled and deionised water, followed by mixtures B and C (for composition, see list below). After thorough mixing, sterile agar cooled to below 80°C was incorporated. Finally, mixture D was added, and the artificial diet poured into trays, which were covered with polythene and stored at 4°C. With the exception of the formaldehyde solution and Wessons salts, all compounds were kept at 4°C.

Table. 23. Composition of the artificial diet used for S. littoralis

Mixture A	
Agar	25g
Deionised water	750cm^3
Mixture B	
Casein	44g
Wheat Germ	93.5g
Cholesterol	1.25
Dried Yeast Powder	19g
Methyl-p Hydroxy Benzoate	1.25g
Sucrose	39g
Sorbic Acid	2g
Deionised water	400 cm^3
Potassium Hydroxide, 4M	6.25 cm^3
Mixture C	
Corn oil	2.5 cm^3
Formaldehyde (10% solution)	5.5 cm^3
Sinigrin (1% solution)	5.0 cm^3
Mixture D	
Antibiotic/Vitamin Mixture	7.5g
Choline chloride	1.25
Vitamin Mixture	
Nicotinic acid	5g
Calcium pantothenate	5g
Riboflavin	2.5g
Aneurine hydrochloride	1.25g

Pyridoxine hydrochloride	1.25g
Folic acid	1.25g
D Biotin	0.1g
Cyanocobalamine	0.01g

Antibiotic and Vitamin Mixture

Vitamin mixture	2g
Streptomycin	4g
Aureomycin	36g
Ascorbic acid	80g

Wessons Salt Mixture

Di-Potassium hydrogen phosphate	129g
Calcium carbonate	120g
Sodium chloride	67g
Magnesium sulphate	40.8g
Calcium hydrogen phosphate	30g
Ferric citrate	11g
Magnesium sulphate.$4H_2O$	2g
Potassium iodide	0.32g
Copper sulphate.$5H_2O$	0.12g
Zinc chloride	0.10g

APPENDIX 2. Size of head capsule and weight range used to select S. littoralis larvae for insecticide bioassay.

Larval Instar	Width of Head Capsule (mm)	Weight Range of test insects (mg)
I	0.25 - 0.31	0.029 - 0.51
II	0.43 - 0.50	1.25 - 2.12
III	0.63 - 0.69	1.91 - 11.63
IV	1.25 - 1.30	60.0 - 90.0
V	1.75 - 1.80	200.0 - 250.0
VI	2.75 - 2.8	670.0 - 800.0

APPENDIX 3. Analysis of the principle ions in the haemolymph of sixth instar S. littoralis larvae

Introduction

In order to carry out in vitro investigations it is necessary to bathe isolated tissue in a physiological saline. This solution should ideally provide a similar biochemical environment to that in the intact animal. In practice this is difficult to achieve and salines are normally made up of the major ions, plus glucose which is used to balance the osmotic pressure and act as a respiration substrate. The optimum concentrations of these ions should be similar to the haemolymph but this is not always possible. For example, allowance has to be made for the contribution of proteins and amino acids and other factors such as ion binding.

Materials and Methods

Since no saline has been constructed specifically for S. littoralis, a number of published Lepidoptera salines were evaluated using the following criteria:

(1) Effects on spontaneously contracting muscle such as the heart and midgut.

(2) The rapidity of muscle death as judged by muscle fibres becoming opaque.

(3) The effect on the resting potential of exposed longitudinal muscle fibres was monitored using intracellular microelectrodes connected to a WP Instruments cathode follower, and a Tetronix SB12N Dual Differential Amplifier and oscilloscope.

None of the published salines (see Table 24) were found to be satisfactory. Therefore the haemolymph of S. littoralis, which is the only extracellular fluid circulating in insects, was analysed for the principle inorganic cations and anions.

Table 24. Composition of published Lepidoptera salines

Species	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	NaH_2PO_4	Tris HCl	KMS	Na_2HPO_4	glucose* sucrose+	Osmotic pressure mOsmole	pH	Reference
	mM												
Saturniidae Antheraea pernyi	12	30	3	18	1-5	1-5				130*	283	6.6	Weevers (1966)
Hyalophora cecropia		32	5	5			5			-	260	8.6	Jungreis & Harvey (1975)
Pyralidae Ephestia kuhniella	32-6	32-7	20-6	25-5						250+		7-2	Deitmer & Rathayer, (1976)
Galeria mellonella	35	36	12	16						247*	500	6.6	Belton (1969)
Sphingidae Manduca sexta	25		4	33			150	25		-	-	7.0	Kammer et al., (1978)
Noctuidae Spodoptera littoralis	150	3	3							-	-	6-6	Gammon (1979)
General Lepidopteran saline	4.9	45	3-8	40	5	5			8	34+	-	6-8	Huddart (1966)

Haemolymph from sixth instar larvae was collected on Parafilm from a cut proleg and a 100 µl sample removed using an automatic pipette with a polypropylene tip. The haemolymph was rapidly diluted with 10 cm^3 of cold distilled water to avoid coagulation and samples were kept refrigerated until analysis. Cations were measured using an Instrumentation Laboratory 151 Atomic Absorption/Emission Spectrophotometer. The anions were estimated by titration; chloride by the method of Schales and Schales (1941), sulphate by the method of Rockstein and Heron (1951). The pH was measured using a Pye 79 meter and the osmotic pressure of a 100 µl sample was determined in a Camlab Digital Micro-osmometer.

Discussion

Any analysis of the haemolymph can only be an approximation because insect blood contains significant quantities of proteins and amino acids (Florkin & Jeuniaux, 1974). It is also complicated by other factors such as ion binding and coagulation. Estimates of the contribution from ion binding have varied. Weevers (1966) has suggested that up to 60% of calcium and magnesium is bound in insect haemolymph, whereas other authors consider ion binding much less important (Florkin & Jeuniaux, 1974).

In this study, the whole haemolymph was analysed and the concentration of free ions may therefore have been over estimated. For example, Brady (1967) found ten times more potassium in the hemocytes of P. americana compared to the plasma. Ideally the plasma and hemocytes should be separated before analysis by centrifugation. Unfortunately, it was not possible to prevent coagulation, even with the addition of 2-5% EDTA (wt/vol) - although this has been used successfully in Orthoptera (Brady, 1967). Centrifuging the blood without fixing would have removed both hemocytes and coagulated plasma, underestimating the ionic concentration.

Table 25. Analysis of sixth instar S. littoralis haemolymph

Ion species	Sixth instar S. littoralis (present study) mM	Sixth instar S. littoralis (Neuman & Voss) mM	Sixth instar Prodenia eridaina (Babers, 1938) mM
Na^+	27.1 ± 2.1	30.14	22
K^+	37.8 ± 4.0	35.45	40
Ca^{++}	8.3 ± 1.1	7.07	7
Mg^{++}	55.7 ± 6.8	36.29	7
Cl^-	31.7 ± 1.4	16.35	34
$S^=$	-	9.43	10
$P^=$	58.6 ± 0.2	52.88	39
Osmotic pressure	332 ± 4 mOmole	-	-
pH	6.8	-	-

Composition of S. littoralis saline

Compound	Concentration mM
$CaCl_2.2H_20$	7.07
Na_2 β-glycerophosphate	21.98
$MgSO_4.7\ H_20$	13.47
$MgCl_2.6\ H_20$	26.86
KCl	29.51
Glucose	3.89

pH 6.8 by addition of KH_2PO_4

Estimation of plasma calcium would also have been difficult after the addition of chelating agents. Similarly, it was not easy to separate plasma bound ions before analysis and therefore no attempt was made to do this.

The results obtained (Table25) indicate that S. littoralis has a similar cationic concentration to other holometabolous herbivorous insects (Florkin & Jeuniaux, 1974). The concentrations of magnesium and potassium in S. littoralis were found to be much greater than sodium. Chloride accounts for only a small proportion of anions, the ionic balance being maintained by phosphates which in Lepidoptera are mainly organic (Florkin & Jeuniaux, 1974).

Using the results from the analysis of haemolymph combined with data provided by Neumann and Voss (personal communication) for S. littoralis and a closely related insect S. eridania (Babers, 1938), a series of salines (see Table 24) were prepared and evaluated.

The most effective saline (Table 25) had concentrations of sodium, potassium and calcium similar to the haemolymph, but magnesium was reduced. Ionic balance was maintained by β-glycerophosphate, sulphate and chloride, all at slightly different concentrations to the haemolymph. An inorganic phosphate was used since Lepidoptera are characterised by the presence of these compounds in the blood. Buffering was provided by addition of potassium dihydrogen orthophosphate, which also reduced the pH from 10 to 6.8. Glucose was added as a respiratory substrate and to increase osmotic pressure.

APPENDIX 4. Mortality data for calculation of median lethal doses.

Key to Tables

(1) Concentration of insecticide applied in μg/g body weight by topical application except where an alternative method is indicated.

(2) Mean body weight.

(3) Standard error of body weight.

(4) % mortality after 48 h.

(5) % mortality after 60 h.

Toxicity data for Table 11.

CDM	(1)	Control	580.8	58.7	29.1	5.8	0.5				
Female	(2)	194.0	177.6	175.7	176.4	177.2	175.8				
	(3)	34.7	15.2	15.3	13.8	14.4	15.1				
	(4)	0	60	40	20	0	0				
	(5)	0	80	80	40	40	0				
CDM	(1)	Control	777.0	66.4	37.1	7.5	4.1				
Male	(2)	117.0	135.1	134.6	141.2	139.0	123.2				
	(3)	4.6	14.6	19.1	16.9	15.3	5.05				
	(4)	0	60	60	40	40	0				
	(5)	0	100	100	60	60	20				
CDM.HCl	(1)	Control	1700.2	680.3	64.5	5.6	0.52	0.052	0.0051		
Female	(2)	144.2	150.6	149.7	159.0	190.1	203.8	200.4	206.4		
	(3)	6.9	11.5	9.9	12.3	24.8	19.9	18.5	21.6		
	(4)	0	100	100	100	80	80	0	0		
	(5)	0	100	100	100	100	40	20	0		
CDM.HCl	(1)	Control	1815.9	735.2	73.5	7.5	0.67	0.069	0.0070		
Male	(2)	135.4	140.7	140.2	137.8	135.8	152.1	148.3	144.3		
	(3)	7.1	9.9	9.9	7.9	7.1	9.8	10.42	8.8		
	(4)	0	100	100	40	20	20	0	0		
	(5)	0	100	100	100	100	60	40	0		
Amitraz	(1)	Control	11472.0	10273.9	7240.1	4317.5	3348.5	847.5	705.0	170.6	70.5
Male	(2)	135.0	136.3	116.8	138.1	162.1	149.	147.5	141.8	146.6	147.1
	(3)	7.0	16.0	9.5	16.6	10.0	11.6	6.7	10.4	7.8	11.7
	(4)	0	100	100	90	60	10	10	10	0	0
	(5)	0	100	100	90	100	100	30	20	20	10

Toxicity data for Table 12.

CDM	(1)	Control	1480.9	759.1	597.2	277.3	142.7	71.3	36.0	14.2					
Sixth	(2)	706.3	874.0	682.9	718.6	724.9	705.5	706.1	700.6	713.3					
Instar	(3)	36.4	45.2	66.2	89.4	25.4	28.9	29.1	31.5	41.0					
	(4)	0	80	80	40	20	0	20	0	0					
	(5)	0	100	100	100	60	40	40	10	10					
CDM.HCl	(1)	Control	1222.7	1119.3	915.1	840.3	777.0	536.0	536.0	241.2	230.3	121.0	66.4	30.3	15.1
Sixth	(2)	697.9	678.5	685.9	759.3	759.34	706.6	647.8	799.9	687.2	733.4	701.0	697.1	693.7	964.5
Instar	(3)	25.9	109.0	78.8	45.6	45.6	45.3	4.6	57.2	31.6	25.0	28.4	23.3	21.0	23.2
	(4)	0	100	100	80	40	40	40	40	0	20	0	0	0	0
	(5)	0	100	100	80	60	60	60	40	40	20	0	20	0	0
CDM	(1)	Control	428.4	429.4	43.9	43.9	4.4	4.4	0.5	0.5					
Fifth	(2)	255.4	237.3	228.7	238.8	218.2	228.0	228.6	206.4	277.1					
Instar	(3)	3.3	6.5	0.3	9.6	6.3	11.6	7.1	0.8	0.3					
	(4)		100	90	80	40	10	0	0	0					
	(5)		100	90	80	40	10	0	0	0					
CDM.HCl	(1)	Control	531.9	531.9	236.1	236.1	126.7	126.7	52.3	52.3					
Fifth	(2)	213.2	226.8	228.8	250.0	243.3	250.0	211.9	211.6	243.4					
Instar	(3)	10.5	7.8	4.4	7.7	6.7	4.7	3.5	2.0	2.0					
	(4)	0	80	20	30	10	10	10	40	10					
	(5)	0	80	20	30	10	10	10	40	10					
CDM	(1)	Control	1653.6	1515.2	162.6	150.0	146.8	125.4	146.8	195.4					
Fourth	(2)	68.9	60.5	68.6	61.5	66.7	68.1	79.7	68.1	79.7					
Instar	(3)	11.1	3.91	3.91	3.7	3.7	8.2	8.2	8.2	8.2					
	(4)	0	100	100	100	80	20	0	20	10					
	(5)	0	100	100	100	80	20	0	20	10					
CDM.HCl	(1)	Control	677.3	627.7	318.5	326.5	69.0	54.3	5.3	5.4					
Fourth	(2)	73.4	62.0	66.9	66.0	64.5	77.5	60.9	77.9	79.9					
Instar	(3)	9.1	9.1	9.1	11.7	11.7	1.0	1.0	3.4	3.4					
	(4)	0	100	20	60	40	20	0	20	0					
	(5)	0	100	20	60	40	20	0	20	0					

continued.....

Toxicity data for Table 12 - continued

CDM.HCl	(1)	Control	806.4	607.4	403.2	294.1	11.5	
Sixth	(2)	720.1	732.5	704.1	732.5	719.7	739.7	
Instar	(3)	21.8	15.8	29.0	15.8	21.5	21.1	
Injection	(4)	0	70	50	30	20	20	
	(5)	0	90	50	40	30	20	
CDM.HCL	(1)	Control	2070.0	1860.0	1305.6	831.4	420.8	211.4
Fifth	(2)	196.9	203.0	182.0	193.5	202.3	200.3	199.2
Instar	(3)	10.6	3.8	21.9	12.9	7.4	14.6	13.0
Ingestion	(4)	0	100	70	60	40	40	30
	(5)	0	100	90	60	50	60	50
Amitraz	(1)	Control	11908.0	10469.0	9457.0	7949.8	6019.3	3895.5
Fourth	(2)	63.8	67.2	66.9	63.4	62.3	64.2	63.8
Instar	(3)	0.5	0.8	0.1	1.0	1.0	2.5	4.0
	(4)	0	0	0	0	0	0	0
	(5)	0	20	30	10	10	0	0
CDM	(1)	Control	13.6	6.8	3.9	2.7	2.0	0.7
Suscept-	(2)	76.4	73.4	73.7	76.4	74.5	76.4	74.5
ible	(3)	0.8	1.4	0.9	0.8	0.8	1.5	1.9
Strain	(4)	0	100	100	90	60	70	60
	(5)	0	100	100	100	80	90	60

Toxicity data for Table 13.

CDM.HCl	(1)	Control	77.6	31.1	17.3	6.1	2.9	1.5				
	(2)	124.2	140.1	146.5	138.6	121.3	135.7	108.9				
	(3)	4.9	4.7	4.6	0.6	2.0	4.4	3.6				
	(4)	0	80	80	50	30	30	10				
	(5)	0	100	100	100	50	30	10				
CDM.HCl	(1)	Control	128.6	73.1	2.9	1.6	0.6					
+ P.B.	(2)	182.3	131.1	116.3	131.2	119.7	143.1					
	(3)	7.1	0.8	2.0	0.3	7.7	1.3					
	(4)	0	90	80	35	40	20					
	(5)	0	100	100	90	75	30					
DCDM	(1)	Control	153.4	83.0	69.8	47.0	37.7	14.1	7.9			
	(2)	154.8	130.8		143.9	159.8	132.8	176.4	160.4			
	(3)	3.9	2.1		4.1	0.8	0.6	0.5	6.1			
	(4)	0	70	50	40	20	0	10	10			
	(5)	0	80	50	50	20	10	10	10			
DCDM	(1)	Control	101.9	53.8	30.4	14.2	5.1	2.8	0.6			
+ P.B.	(2)	16.2	185.0	171.5	176.5	198.7	177.7	179.0	204.5			
	(3)	1.2	2.7	15.4	4.3	0.7	0.7	1.6	0.4			
	(4)		100	90	10	90	60	10	0			
	(5)		100	100	100	100	90	40	10			
Amitraz	(1)	Control	12199.0	8906.9	7720.1	6428.9	5750.6	3752.2	3072.4	2819.6	2326.3	1580.4	716.4
	(2)	182.3	144.6	168.7	162.1	183.3	174.0	167.0	162.8	142.7	161.6	158.2	174.8
	(3)	7.0	5.7	3.2	1.8	7.9	1.5	4.0	0.5	5.0	3.6	0.4	3.4
	(4)	0	100	100	87	20	20	20	20	20	20	10	0
	(5)	0	100	100	100	80	70	70	40	40	20	10*	0
Amitraz	(1)	Control	6569.3	5965.0	5272.9	4230.3	3061.7	1850.7	568.1	273.8	157.8	64.5	
+ P.B.	(2)	124.0	191.4	172.8	142.9	152.8	163.3	135.2	176.1	183.4	158.7	155.1	
	(3)	3.9	6.6	13.3	4.3	12.8	0.9	1.8	1.1	5.4	1.5	0.05	
	(4)	0	90	80	90	90	80	70	50	50	50	0	
	(5)	0	100	100	100	100	100	100	80	70	50	40	

Toxicity data for Table 14.

CDM.HCl	(1)	Control	77.6	31.1	17.3	6.1	2.9	1.5	
Topical	(2)	124.2	140.1	146.5	138.6	121.3	135.7	108.9	
	(3)	4.9	4.7	4.6	0.6	2.0	4.4	3.6	
	(4)	0	80	80	50	30	30	10	
	(5)	0	100	100	100	50	30	10	
CDM.HCl	(1)	Control	45.4	31.9	15.5	6.1	1.4	0.7	0.04
Injec-	(2)	184.9	130.9	135.2	139.0	144.6	127.9	192.1	136.4
tion	(3)	2.61	0.3	1.9	3.1	4.3	0.5	3.9	2.0
	(4)	0	90	60	30	10	10	10	0
	(5)	0	100	70	60	60	20	20	0

CDM	(1)	Control	1812.7	922.8	622.0	457.4	192.0	82.1	
Topical	(2)	112.4	121.9	131.9	110.0	120.6	108.4	110.4	
	(3)	0.3	0.5	6.5	3.8	1.2	0.7	1.1	
	(4)	0	70	50	20	30	30	30	
	(5)	0	90	90	80	90	90	40	

CDM	(1)	Control	1380.8	865.8	673.1	63.9	40.5	39.7	15.1	6.3
Injec-	(2)	112.4	144.9	173.5	148.6	160.4	185.4	127.1	165.5	160.5
tion		±	±	±	±	±	±	±	±	±
	(3)	0.3	0.7	2.7	0.4	11.1	0.8	5.2	2.8	8.2
	(4)	0	60	40	60	50	10	0	0	0
	(5)	0	80	50	60	50	20	30	0	0

Toxicity data for Table 15.

DDT	(1)	Control	4615.0	3727.3	2433.7	298.2	5.8	1.2	0.6			
	(2)	88.3	86.7	80.5	82.2	83.8	86.8	84.6	88.2			
	(3)	0.8	0.7	1.3	1.3	1.6	2.6	1.2	2.3			
	(4)	0	70	40	60	20	10	10	10			
	(5)	0	70	70	60	30	20	10	10			
CARB-ARYL	(1)	Control	3348.7	2951.7	2376.0	1674.2	1171.2	118.9	58.1	6.1	5.9	1.2
	(2)	84.9	89.6	84.7	84.2	89.6	85.4	84.3	86.0	82.0	85.0	80.5
	(3)	0.9	0.7	0.4	0.7	0.5	0.7	1.1	1.6	0.7	3.4	3.6
	(4)	0	30	60	50	40	50	10	0	10	0	0
	(5)	0	60	60	50	50	60	10	10	20	0	0
Perme-thrin	(1)	Control	30.2	12.2	6.2	1.2	0.62	0.3	0.12	0.06	0.01	
	(2)	88.1	80.4	82.1	82.8	85.0	81.7	82.9	82.2	84.5	86.4	
	(3)	2.0	0.8	0.1	0.9	1.0	7.1	0.5	0.9	1.1	0.9	
	(4)	0	100	100	100	70	60	30	1	0	0	
	(5)	0	100	100	100	80	60	30	10	10	10	

Toxicity data for Table 16.

CDM	(1)	Control	711.0	60.1	5.1	0.54	0.054
Female	(2)	168.0	146.7	172.3	201.3	193.2	193.4
Acetone	(3)	10.5	13.5	14.8	18.4	16.4	15.2
	(4)	0	100	60	50	0	0
	(5)	0	100	90	90	40	0
CDM	(1)	Control	5487.4	499.8	56.5	5.6	0.57
Female	(2)	186.9	186.8	204.3	183.5	185.4	184.5
Hexa-	(3)	11.5	15.0	14.5	17.0	17.0	18.2
decane	(4)	0	100	60	20	0	0
	(5)	0	100	100	60	20	0

CDM	(1)	Control	795.6	73.3	7.0	0.74	0.07
Male	(2)	140.7	126.3	139.2	143.8	138.2	153.3
Acetone	(3)	8.9	2.9	6.7	3.0	6.5	15.8
	(4)	0	100	50	20	0	0
	(5)	0	100	100	40	40	0
CDM	(1)	Control	6308.5	634.7	63.4	6.4	0.64
Male	(2)	151.8	164.0	162.3	161.8	159.7	157.8
Hexa-	(3)	22.9	15.1	14.3	13.1	10.4	9.1
decane	(4)	0	100	100	60	20	0
	(5)	0	100	100	80	20	0

Toxicity data for Table 17.

CDM	(1)	Control	594.6	462.0	318.1	151.9	111.7	80.4	37.8	18.2
+ P.B.	(2)	67.7	67.3	65.0	62.9	65.8	67.1	63.2	66.2	68.6
Fourth	(3)	2.4	0.2	3.6	3.2	0.7	2.6	2.1	0.8	1.0
Instar	(4)	0	100	100	70	50	60	40	10	10
	(5)	0	100	100	80	80	70	80	20	20
DCDM	(1)	Control	1462.1	1053.7	777.1	304.0	142.8	108.9	65.6	36.8
	(2)	71.3	68.4	66.4	64.4	66.1	70.7	69.4	76.3	69.1
Fourth	(3)	9.3	1.3	1.1	8.3	4.4	6.9	1.0	0.3	1.8
Instar	(4)	0	40	20	20	10	0	0	0	0
	(5)	0	40	20	20	10	0	0	0	0
DCDM	(1)	Control	2441.0	2037.0	1574.3	1086.3	695.0	366.0	288.0	
+ P.B.	(2)	62.3	63.5	62.2	62.1	69.9	72.0	68.2	69.6	
Fourth	(3)	0.2	0.2	0.1	5.0	0.3	2.1	1.1	0.5	
Instar	(4)	0	60	20	40	30	10	0	0	
	(5)	0	60	20	40	40	20	0	0	

APPENDIX 5. Thin layer chromatography of labelled synephrine.

Tissue	% of total radioactivity co-chromatographing with authentic synephrine		
	Solvent (1)	Solvent (2)	Solvent (3)
Ventral nerve cord	98%	97%	97%
Salivary glands	97%	98%	91%
Malpighian tubules	97%	97%	96%
Fat body	96%	96%	97%
Muscle	93%	92%	93%
Testis	91%	90%	92%
Haemolymph	92%	92%	94%

Solvent (1) propan-1-ol:ammonia:water 80:19:10

Solvent (2) butan-1-ol saturated with 1N HCl.

Solvent (3) butan-1-ol:acetic acid:water 4:1:1

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